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Identification of menaquinone-4 (vitamin K2) target genes in bovine endometrial epithelial cells *in vitro*

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Abstract

The effect of vitamin K on bovine endometrial epithelial cells has not been thoroughly investigated. The objective of this study was to examine the effect of the biologically active form of vitamin K, menaquinone-4, on gene expression in bovine endometrial epithelial cells. First, we examined the mRNA and protein expression levels of UBIAD1, a menaquinone-4 biosynthetic enzyme. Second, we screened for potential target genes of menaquinone-4 in bovine endometrial epithelial cells using RNA-sequencing. We found 50 differentially expressed genes; 42 were upregulated, and 8 were downregulated. Among them, a dose-dependent response to menaquinone-4 was observed for the top three upregulated (*TRIB3*, *IL6*, and *TNFAIP3*) and downregulated (*CDC6*, *ORC1*, and *RRM2*) genes. It has been suggested that these genes play important roles in reproductive events. In addition, *GDF15* and *VEGFA*, which are important for cellular functions as they are commonly involved in pathways, such as positive regulation of cell communication, cell differentiation, and positive regulation of MAPK cascade, were upregulated in endometrial epithelial cells by menaquinone-4 treatment. To the best of our knowledge, this is the first study showing the expression of UBIAD1 in the bovine uterus. Moreover, the study determined menaquinone-4 target genes in bovine endometrial epithelial cells, which may positively affect pregnancy with alteration of gene expression in cattle uterus.

Keywords: cow, endometrial epithelial cell, gene expression, menaquinone-4, vitamin K

1. Introduction

Vitamin K (VK) plays essential roles in the blood coagulation system [1]. The VK family consists of VK1 (phylloquinone), VK2 (menaquinones, MKs), and VK3 (menadione). Vitamin K1 is found in plants, VK2 refers to a group of MKs characterized by different side-chain lengths, and VK3 is either chemically synthesized or an intermediate of MK-4 synthesis. The various forms of MKs are named MK-n, where “n” refers to the number of isoprenoid residues in the side chain [2]. Dietary VK1 is endogenously converted to MK-4 [3, 4], which is the most common form of short-chain MK and the

51 functional VK2 in animal tissues [5, 6]. Long-chain MKs (MK-5 to MK-13) are synthesized by bacteria
52 [2]. The presence of MK-4 in various body tissues suggests that it plays physiological roles. In addition
53 to blood coagulation, VKs are involved in several processes, including bone metabolism, cell
54 proliferation, and anti-inflammation [7–11].

55 To date, extensive studies have been conducted on vitamins A, D, and E because of their
56 frequent deficiency in dairy cows [12, 13]. However, few studies have evaluated VK and its functions.
57 In cows, VK2 is synthesized by rumen microbes to meet nutritional requirements. We recently showed
58 that MK-4 positively influences the activation of peripheral blood mononuclear cells and potentially
59 activates immune functions in dairy cows [14]. Therefore, examination of VK functions in specific
60 tissues may be significant for understanding the physiological roles of VKs in ruminants.

61 In MK-4 biosynthesis, the phytyl and prenyl groups of VK1 and VK2 dissociate from the
62 naphthoquinone ring to form VK3, with no side-chain structures. Then, geranylgeranyl diphosphate
63 (derived from the mevalonate pathway) is covalently bound to VK3 by the prenyltransferase enzyme,
64 *UbiA* prenyltransferase domain-containing 1 (UBIAD1), to produce MK-4 [4, 15]. UBIAD1 expression
65 is detected in multiple organs, including the mouse uterus [4]. In mice, VK1 and VK3 play a role in
66 uterine myometrial contraction [16, 17]. Zhang et al. [16] found that VK3 reduced the effectiveness of
67 prostaglandin F2α-induced uterine contraction; thus, VK3 may be a promising therapeutic strategy for
68 myometrial contractile complications, such as implantation failure, dysmenorrhea, and preterm birth.
69 Similarly, VK1 attenuated oxytocin-induced uterine contractions and may contribute to the
70 management and treatment of reproductive disorders [17]. On the other hand, to the best of our
71 knowledge, UBIAD1 expression and the effects of MKs have not yet been studied in mammalian
72 endometrial epithelial cells, including ruminants.

73 The endometrium epithelial cells play a wide range of important roles such as the prevention
74 of microbes, production of growth factors/cytokines, and reception of embryos. In this study, we
75 hypothesized that if UBIAD1 is expressed in the bovine endometrium, the produced MK-4 may play a
76 role in this tissue. VKs play roles in a wide range of biological processes beyond blood coagulation;
77 therefore, identifying their target genes is critical for a better understanding of their organization. Thus,

78 we aimed to reveal the UBIAD1 expression in bovine endometrial epithelial cells and examine the
79 effects of the biologically active form of VK, MK-4, on gene expression. Our findings may reveal
80 changes in gene expression caused by MK-4 and provide insights into the regulation of the intrauterine
81 environment.
82
83 **2. Materials and Methods**
84 **2.1. Collection of bovine endometrial tissues**
85 Uterine tissues of non-pregnant healthy Holstein cows (approximately 30 to 100 month old)
86 were obtained from a local slaughterhouse (Hokkaido Hayakita Meat Inspection Center, Hokkaido,
87 Japan; and Nichiro Chikusan Co., Ltd., Hokkaido, Japan). The collection of bovine endometrial tissues
88 has been described previously [18]. Briefly, endometrial tissues of the intercaruncular area were
89 dissected from the uterine horn, ipsilateral to the corpus luteum, and cut into small pieces (3 × 3 mm).
90 The collected endometrial tissues were classified into early, mid, and late estrous stages, according to
91 the luteal stages (n = 8/stage), to examine *UBIAD1* expression. For cell culture, tissue samples were
92 used for cell culture experiments at random without distinguishing the stages. The tissue used were;
93 early luteal phase, n = 4; middle luteal phase, n = 2; late luteal phase, n = 4.
94
95
96 **2.2. Culturing of bovine endometrial epithelial cells**
97 The culturing method of bovine endometrial epithelial cells from uterine tissues has been
98 described previously [19]. Briefly, the epithelial cells were cultured in Dulbecco's modified Eagle's
99 medium (DMEM; Wako, Osaka, Japan) supplemented with 5% (v/v) fetal bovine serum (FBS; BioWest,
100 Funakoshi Co., Ltd., Tokyo, Japan) and antibiotic-antimycotic solution (Thermo Fisher Scientific,
101 Waltham, MA, USA) at 38.5 °C with 5% CO_2. The cells were used within ten passages. Human
102 endometrial epithelial cell line HHUA [20] and OMC9 [21] were obtained from Riken Cell Bank
103 (Tsukuba, Japan) and cultured in DMEM with 10% FBS.
104

105 **2.3. Preparation and treatment of MK-4**

106 Cells were cultured on 60-mm collagen-coated dishes (IWAKI AGC TECHNO GLASS Co.
107 Ltd., Shizuoka, Japan) for RNA extraction. Menaquinone-4 (Wako) was dissolved in 99.5% (v/v)
108 ethanol (10 mM) as a stock solution and diluted with the culture medium. The cells were cultured in
109 DMEM containing 5% FBS and an antibiotic-antimycotic solution with MK-4 (1, 10, 50, or 100 μM)
110 or without (cultured medium only, as the control, or 1% ethanol, as the vehicle control; equivalent
111 amount of ethanol to the 100 μM MK-4 treatment). The cells were cultured for 12, 24, and 48 h under
112 a humidified atmosphere of 5% CO_2 at 38.5 °C. Two dishes of samples from each group (control or
113 MK-4 treatment) were used for analysis. Ten independent experiments (replicates) were conducted.

114 Cell proliferation assays were conducted using the Cell Counting Kit-8 (Dojindo, Kumamoto,
115 Japan) according to the manufacturer's instructions. Due to the lack of knowledge of MK-4
116 concentration in bovine uterine tissue, multiple concentrations were examined based on the findings
117 from previous *in vitro* cell culture systems [22, 23]. To confirm the absence of toxicity caused by the
118 MK-4 treatment, cell proliferation at multiple concentrations (1, 10, 50, or 100 μM) was examined at
119 the 48 h time point. The cells were seeded onto a 96-well plate (1.0×10^4 cells/well/200 μL) for
120 proliferation assays. The stimulated index (SI) was calculated as the ratio of the average absorbance
121 value from three wells containing MK-4-treated cells relative to that from three wells containing non-
122 treated cells. Ten independent experiments were conducted.

123

124 **2.4. RNA extraction and analysis**

125 Total RNA was extracted from endometrial epithelial tissue pieces (3 × 3 mm) (from the
126 UBIAD1 detection experiment in 2.1) or endometrial epithelial cells (from the MK-4 treatment
127 experiment in 2.3) using NucleoSpin RNA Plus (Takara Bio Ltd., Shiga, Japan) according to the
128 manufacturer's protocol. For quantitative real-time PCR (qPCR) analysis, the total RNA isolated (500
129 ng) was reverse transcribed to cDNA using the ReverTra Ace qPCR RT Master Mix with gDNA
130 Remover (Toyobo, Osaka, Japan). The cDNA reaction mixture was diluted with molecular biology-
131 grade water five times, and 1 μL was taken for each amplification reaction. Target gene expression

levels were determined by qPCR using a LightCycler 96 (Roche Diagnostics, Basel, Switzerland) and THUNDERBIRD™ SYBR qPCR Mix (Toyobo) with 0.5 μM (final concentration) of the primers listed in Table 1. The thermal cycling conditions were as follows: 1 cycle at 95 °C for 30 s, followed by 50 cycles at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 30 s. Relative mRNA abundance was calculated based on the expression levels of bovine *H2AFZ*, which was used as a reference gene as it shows stable expression in the bovine endometrium [24, 25], and it was relatively stable under MK-4 treatment in our preliminary observations. Each run was completed with a melting curve analysis to confirm specific amplification and no primer dimer formation. All experiments were performed following the guidelines of Minimum Information for Publication of Quantitative Real-Time PCR Experiments [26].

2.5. RNA-sequencing and data analysis

In the preliminary examination, 1 to 100 μM of MK-4 did not show significant toxicity (Supplementary Figure 1); thus, the MK-4 treatment for RNA-sequencing was performed at a maximum concentration of 100 μM. Total RNA was extracted from bovine endometrial epithelial cells with or without MK-4 treatment (100 μM for 48 h; n = 4 per group) using NucleoSpin RNA Plus (Takara Bio UK Ltd., Shiga, Japan). Genome-Lead Corporation (Kagawa, Japan) performed library construction and RNA-sequencing~~.~~, to whom we sent 100 μL of RNA solution adjusted to 30 ng/μL for analysis. Libraries were constructed using KAPA mRNA Capture Kit (Roche) and MGIEasy RNA Directional Library Prep Set (MGI). Sequencing was performed in 150-bp paired-end format on a DNBSEQ-G400RS system. Sequencing reads were mapped to the bovine genome (ARS-UCD1.2) using HISAT2 [~~25~~27]. The data analyses were based on clean data using iDEP [28]. All RNA-sequencing data were deposited in the DDBJ data bank (https://www.ddbj.nig.ac.jp) (accession number: DRA014943).

2.6. Western blotting

To detect the UBIAD1 protein, endometrial lysates were prepared in Radioimmunoprecipitation assay (RIPA) buffer (Wako). The lysates were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane using an iBlot Gel Transfer

159 Device system (Invitrogen). After blocking with PVDF blocking buffer (CanGetSignal, Toyobo), the
160 membranes were treated with a rabbit polyclonal anti-UBIAD1 antibody (ab191691, Abcam, 1 μg/mL)
161 or mouse monoclonal anti-beta actin (ACTB) antibody (internal control, 66009-1-Ig, Proteintech, IL,
162 USA, 1:5000), for 1 h at room temperature. Blotting was performed independently for each antibody
163 using the same sample (n = 3 for each antibody). Proteins were detected using the following two
164 secondary antibodies: (1) Amersham ECL Anti-rabbit IgG, Horseradish peroxidase-Linked Species-
165 Specific Whole Antibody (from donkey), or (2) Amersham ECL Anti-Mouse IgG, Horseradish
166 peroxidase-Linked Species-Specific Whole Antibody (from sheep), and EzWestLumi Plus (ATTO).
167 Protein bands were visualized using a chemiluminescence analyzer (LumiCube, Liponics Inc., Tokyo,
168 Japan), and the band intensities were quantified using ImageJ software (ImageJ 1.53k, NIH, Bethesda,
169 MD, USA).
170
171 **2.7. Immunocytochemistry**
172 Immunohistochemical analyses were performed on 10-μm frozen sections of bovine uterine
173 tissues using a polyclonal rabbit anti-UBIAD1 antibody (ab191691, Abcam, 5 μg/mL) and ImmPRESS
174 Universal (Horse Anti-Mouse/Anti-Rabbit IgG) PLUS Polymer Kit (Vector Laboratories, Inc.
175 Burlingame, CA, USA). The sections were examined using LAS X with DMi8 (Leica, Tokyo, Japan).
176
177 **2.8. Statistical analysis**
178 Results are expressed as the mean ± standard error of the mean (SEM). Data were analyzed
179 using the R software package (version 3.6.2) (https://www.r-project.org/). Data were analyzed using
180 one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple comparisons
181 with each group. Differences with P-values < 0.05 were considered statistically significant.
182
183 **3. Results**
184 **3.1. Expression of UBIAD1 in bovine endometria**
185 The expression of *UBIAD1* mRNA was detected in bovine endometria in the early, mid, and

186 late estrous stages. No significant differences were detected among the stages (P > 0.05), indicating a
187 stable expression. Likewise, western blotting and immunostaining revealed that the UBIAD1 protein
188 was detectable throughout the estrous stages, and no significant differences were detected among the
189 stages (P > 0.05) (Figure 1).
190
191 **3.2. Effects of MK-4 on gene expression of bovine endometrial epithelial cells**
192 To identify target candidate genes of MK-4 in bovine uterine endometrial epithelial cells, we
193 first conducted RNA-sequencing. Based on the RNA-sequencing analysis using iDEP, 50 differentially
194 expressed genes (DEGs), including 42 upregulated and eight downregulated genes, were found as
195 potential targets of MK-4 (Figure 2). All the DEGs are listed in Table 2. Subsequently, qPCR ensured
196 the expression of the top three upregulated (*TRIB3*, *IL6*, and *TNFAIP3*) and downregulated (*CDC6*,
197 *ORC1*, and *RRM2*) genes in bovine endometrial epithelial cells. Furthermore, a dose- and time-
198 dependent increase or decrease in the expression of these genes by MK-4 was confirmed (Figure 3).
199 *UBIAD1* expression was not affected by the MK-4 treatment (100 μM, 48 h) (Supplementary Figure 2).
200
201 **3.3. Enriched Gene Ontology terms in genes upregulated by MK-4 treatment**
202 Process terms enriched in the DEGs were analyzed by iDEP (Table 3). Fifteen pathways were
203 found to be enriched: response to organic substance, cellular response to organic substance, apoptotic
204 process, programmed cell death, cell death, developmental process, response to chemical, cellular
205 response to chemical stimulus, response to peptide, response to oxygen-containing compound, cellular
206 response to oxygen-containing compound, positive regulation of cell communication, positive
207 regulation of signaling, cell differentiation, and positive regulation of MAPK cascade. Although gene
208 expression in the apoptotic-related pathways (apoptotic process, programmed cell death, and cell death)
209 was also increased, the expression of effector caspases (*CASP3* and *CASP7*) was not affected
210 (Supplementary Figure 3). Cell viability was also not significantly adversely affected by MK-4
211 treatment (Supplementary Figure 1), as mentioned above.
212

213 **3.4. Key factors in the upregulated pathways**

214 The top three genes upregulated by MK-4 (*TRIB3*, *IL6*, and *TNFAIP3*) were found in almost
215 all enhanced pathways. In addition, we focused on growth factor genes, *GDF15* and *VEGFA*. These
216 genes were commonly involved in pathways, such as the positive regulation of cell communication, cell
217 differentiation, and positive regulation of MAPK cascade pathways, which are important for cellular
218 functions. A dose- and time-dependent increase in the expression of *GDF15* and *VEGFA* by MK-4 in
219 bovine endometrial epithelial cells was confirmed by qPCR (Figure 4). Furthermore, MK-4 treatment
220 also increased the expression of these genes in human endometrium-derived HHUA and OMC9 cells.

221

222 **4. Discussion**

223 UBIAD1 expression is detected in multiple mouse organs, including the uterus [4]. In mice,
224 VK_1 and VK_3 play roles in uterine myometrial contraction [16, 17]. To the best of our knowledge, this
225 is the first report showing UBIAD1 expression in bovine endometrial tissue. The presence of UBIAD1
226 suggests that uterine tissue is one of MK-4's conversion sites. In this study, we confirmed the stable
227 expression of UBIAD1 in bovine endometrial epithelial tissues, which was not affected by the estrous
228 cycle or MK-4 treatment. Although the factors affecting UBIAD1 expression and regulation of MK-4
229 conversion activity require further investigation, UBIAD1 may commonly be expressed in mammalian
230 endometrial tissues.

231 Possible targets of MK-4 in bovine endometrial epithelial cells were explored using RNA-
232 sequencing analysis. We found 42 upregulated and 8 downregulated DEGs. The top three upregulated
233 genes were *TRIB3*, *IL6*, and *TNFAIP3*, and the top three downregulated genes were *CDC6*, *ORC1*, and
234 *RRM2*. Genes upregulated by MK-4 treatment included *GDF15* and *TRIB3*, which were previously
235 reported as targets of MK-4, although in different animals and tissues [29]. Furthermore, 15 pathways
236 were elevated in bovine endometrial epithelial cells by MK-4 treatment. The top three upregulated genes
237 were found in almost all these pathways. Thus, these factors are suggested to play an important role in
238 reproductive events, and the intrauterine environment may be regulated through their modulation.

239 TRIB proteins are involved in various cellular events such as inflammation, differentiation,

240 and proliferation [30–32]. The TRIB family members regulate the innate immune system by interacting
241 with the toll-like receptor (TLR)-mediated nuclear factor kappa-B (NF-κB) signaling pathways [33–
242 35], suggesting modulation of the immune environment during implantation and pregnancy [30]. In
243 addition, it was reported that the CHOP/TRIB3/AKT/mTOR axis is involved in the regulation of the
244 invasive potential of normal human endometrial cells [36], while TRIB3 inhibited proliferation and
245 migration and promoted apoptosis of endometrial cancer cells [37]. These findings suggest that TRIB3
246 is important for normal endometrial cell growth.

247 The multifunctional cytokine IL-6 has an important role in reproduction, particularly
248 during early embryonic development and implantation [38–41]. In ruminants, IL-6 stimulates protease
249 production, and it is thought to be involved in placentation [42]. IL-6 is also produced by the conceptus
250 in many species, including bovine preimplantation embryos [43, 44]. Although differences in the
251 function of IL-6 from embryonic and endometrial sources are not clear, the importance of adequate IL-
252 6 production during gestation is suggested.

253 *TNFAIP3* encodes the A20 protein, an important regulator of NF-κB-mediated inflammatory
254 signaling, and defects in this protein in immune cells cause systemic inflammation and autoimmunity
255 [45, 46]. Although findings in the uterus are very limited, A20 is expressed in the rat uterus and human
256 endometrial cells [47, 48], suggesting its role in regulating the immune environment along with other
257 cytokines.

258 ORC1 and CDC6 play a role in cell-cycle DNA replication [49]. In humans, CDC6 expression
259 is a potential marker for high-grade squamous and glandular dysplasia of the cervix [50, 51]. In mice,
260 RRM2 is a possible marker for cervical cancer [52, 53]. In contrast, however, RRM2 was important for
261 inducing cell proliferation and decidualization in the mouse uterus [54]. The role of these factors in the
262 bovine endometrium is unknown and remains to be validated.

263 Vitamin K2 shows an inhibitory effect on the growth of tumor cells, including hepatoma cells
264 [55]. Furthermore, VK2 induces an apoptotic effect on leukemia cell lines [56] and fibroblast-like
265 synoviocytes [57]. However, in the present study on primary endometrial epithelial cells, the expression
266 of effector caspases (*CASP3* and *CASP7*) was not affected, and cell viability was not significantly

267 adversely affected by MK-4 treatment. Thus, MK-4 does not appear to be toxic in endometrial epithelial
268 cells at the doses tested; however, it will be necessary to carefully assess the extent to which *in vitro*
269 trials on cells reflect the situation *in vivo*. Furthermore, the causes and detailed mechanisms underlying
270 the different effects of MK-4 treatment on cell proliferation in different cell types need to be further
271 investigated.

272 The growth factors GDF15 and VEGFA were found among the pathways enhanced by MK-4
273 treatment. GDF15, also known as macrophage inhibitory cytokine-1, is found in placental trophoblast
274 cells, decidua, and fetal membranes in humans [58, 59], suggesting that GDF15 is involved in the
275 establishment and maintenance of pregnancy at the maternal-fetal interface. VEGFA is expressed in
276 bovine placental trophoblasts and acts to modulate steroidogenesis during gestation [60], suggesting
277 that VEGF is an important regulator of placental development and function. In this study, the expression
278 of both *GDF15* and *VEGFA* was upregulated by MK-4 in bovine as well as human endometrium-
279 derived cells. This suggests that these genes are the common targets of MK-4 in the mammalian uterus,
280 not only in the bovine uterus.

281

282 We recently reported that MK-4 levels in body fluid could be increased by supplemental
283 feeding with VK3 [61]. Therefore, clarifying the physiological function of MK-4 and regulation of MK-
284 4 concentrations via feeding would be useful for better management of health conditions, including
285 those affecting the uterus in mammals. The current study was performed using an *in vitro* culture system,
286 but further validation is needed to determine the actual MK-4 concentration and variation in uterine
287 tissue. In addition, we did not compare pregnant and non-pregnant conditions; thus, further studies are
288 required to gain a better understanding of the function of MK-4 and these downstream candidate target
289 genes in implantation and pregnancy.

290

291 In conclusion, this study confirmed the expression of UBIAD1 in the bovine uterus and found
292 candidate target genes for MK-4 for the first time. In particular, GDF15 and VEGFA were found to be
293 included in most of the pathways enhanced by MK-4 treatment, suggesting their importance. Further

294 studies are needed to expand the understanding of these MK-4 target genes and enhanced biological
295 pathways and their influence on mammalian pregnancy.
296
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299
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304 **Author contributions**
305 H. B. designed the experiments, conducted the experiments, and analyzed the data. M.K. and M.T.
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504

505

506 **Figure legends**

507 **Figure 1.** Expression of UBIAD1 in the bovine endometrium

508 (A) Data represent the *UBIAD1* mRNA expression levels relative to those of the internal control gene,
509 *H2AFZ*. Data are presented as means ± standard error of the mean (SEM). No significant differences
510 were observed among the stages of expression based on the Tukey–Kramer test (n = 8 per group). E:
511 Early stage, M: Middle stage, L: Late stage.

512 (B) Left: UBIAD1 protein levels in Early (E), Middle (M), and Late (L) stages (n = 3 each day) in
513 bovine endometrial epithelial tissues were examined by western blotting. A Rabbit polyclonal anti-
514 UBIAD1 antibody was used to detect the protein and ACTB was used as an internal control.
515 Representative PVDF membrane data from one of the three independent experiments are shown.
516 Right: Densitometric quantitation was performed using the ImageJ software. The relative densities of
517 the blots were determined by normalization against ACTB levels.

518 (C) Immunohistochemical detection of UBIAD1 protein in bovine endometrial epithelial tissues was
519 carried out. Instead of the rabbit polyclonal anti-UBIAD1 antibody, pre-immune serum was used as a
520 negative control. Representative micrographs (Middle stage) from three experiments are shown. Bar =
521 150 μm.

522

523 **Figure 2.** Identification of MK-4 target genes via RNA-sequencing.

524 (A) Heatmap, (B) Volcano plot, and (C) MA plot of differentially expressed genes. (D) Left: Top 3
525 upregulated genes by iDEP analysis. Right: Data represent *TRIB3, IL6,* and *TNBFAIP3* mRNA
526 expression levels relative to those of *H2AFZ*. Data are presented as means ± SEM. Different letters
527 indicate significant differences between groups ($P < 0.05$). (E) Left: Top 3 downregulated genes by
528 iDEP analysis. Right: Data represent *CDC6, ORC1,* and *RRM2* mRNA expression levels relative to
529 those of *H2AFZ*. Data are presented as means ± SEM. Different letters indicate significant differences
530 between groups ($P < 0.05$).

531

532 **Figure 3.** Dose- and time- dependent response to MK-4 treatment.

533 (A) Expression patterns of upregulated (*TRIB3, IL6,* and *TNBFAIP3*) and downregulated (*CDC6, ORC1,*
534 and *RRM2*) genes with different dosages of MK-4 (1, 10, 50, or 100 μM) for 48 h. Data are presented
535 as means ± SEM. Different letters indicate significant differences between groups ($P < 0.05$). (B)
536 Expression of upregulated (*TRIB3, IL6,* and *TNBFAIP3*) and downregulated (*CDC6, ORC1,* and *RRM2*)
537 genes with different treatment times of MK-4. Data are presented as means ± SEM. Different letters
538 indicate significant differences between groups ($P < 0.05$).

539

540 **Figure 4.** Expression of *GDF15* and *VEGFA* after MK-4 treatment in endometrial epithelial cells.

541 (A) Expression levels of *GDF15* and *VEGFA* genes induced by MK-4 as identified by iDEP. (B)
542 Expression patterns of *GDF15* and *VEGFA* genes with different dosages of MK-4. Data are presented
543 as means ± SEM. Different letters indicate significant differences between groups ($P < 0.05$). (C)
544 Expression of *GDF15* and *VEGFA* genes with different treatment times of MK-4. Data are presented
545 as means ± SEM. Different letters indicate significant differences between groups ($P < 0.05$). Data
546 represent *GDF15* and *VEGFA* mRNA expression levels after MK-4 treatment in human endometrium-
547 derived HHUA (D) and OMC9 (E) cells. Data are presented as means ± SEM. Different letters indicate
548 significant differences between groups ($P < 0.05$).

549

550 **Supplemental Figure 1.** The proliferation of bovine endometrial epithelial cells with MK-4 treatment.
551 The cells were treated with different dosages (1, 10, 50, or 100 μM) of MK-4 for 48 h. SI: stimulation
552 index (absorbance from stimulated wells/absorbance from non-stimulated wells). Data are presented as
553 means ± SEM.

554

555 **Supplemental Figure 2.** Expression of *UBIAD1* after MK-4 treatment in endometrial epithelial cells.

556 (A) Expression levels of the *UBIAD1* gene after MK-4 treatment found by iDEP. (B) Relative
557 expression of the *UBIAD1* mRNA level after MK-4 treatment. Data are presented as means ± SEM.

558

559 **Supplemental Figure 3.** Enriched GO terms in genes up- and downregulated by MK-4 treatment.

560 Visualization of the relationship among enriched GO terms using (A) hierarchical clustering tree and

561 (B) network analysis by iDEP. (C) Expression levels of *CASP3* and *CASP7* genes after MK-4 treatment

562 found by iDEP. (B) Relative expression of *CASP3* and *CASP7* mRNA levels after MK-4 treatment.

563 Data are presented as means ± SEM.

564

Table 1. Primers for real time PCR

Name (GenBank accession No.)	Sequence (5'-3')	Product length (bp)
UBIAD1	F: GCTTGCCTCTACTGTCTGTC	114
(XM_002694056.5)	R: AGCCAGGTACTTGAGTCCAAT	
Upregulated genes		
TRIB3	F: CTGAGTGTTCCCGCTGGGTC	184
(NM_001076103)	R: GGTGGTGGGTTCAGGGTTAG	
IL6	F: TAAGCGCATGGTCGACAAAA	150
(EU276071)	R: TTGAACCCAGATTGGAAGCAT	
TNFAIP3	F: CACGCTGTGTTTCATCGAGT	148
(XM_005210987.4)	R: GTATCCTTCGAACACGGTGC	
Downregulated genes		
CDC6	F: CCACAGCTGTTGAACTTCCC	144
(NM_001192407.1)	R: TCCCGAAACAGCAGAGACTT	
ORC1	F: CCGTTCTGGAACAGAGCTTCC	144
(NM_001014918.1)	R: TCTCCGACATGGTGGGGTA	
RRM2	F: TGGCTCAAGAAACGAGGACT	125
(NM_001244181.1)	R: TCCGAAGGTTTGTGCAACAG	
Growth factor genes		
bovine GDF15	F: CAGACCTGGGAAGACTCGAA	134
(NM_001206298.2)	R: GGGAGCCCCTCAGTTAAGTT	
human GDF15	F: CAGAGCTGGGAAGATTCGAA	115
(NM_001206298.2)	R: GGGAGCCCCTCGGGAAGGGC	
bovine VEGFA	F: CCATGAACTTTCTGCTCTCTTGG	133
(NM_174216.2)	R: TCCATGAACTCCACCACTTCG	[62]
human VEGFA	F: CCATGAACTTTCTGCTCTCTTGG	136
(M_001025366.3)	R: TCCATGAACTCCACCACTTCG	
Apotosis related genes		
CASP3	F: AGCGTCGTAGCTGAACGTAA	122
(NM_001077840)	R: CCAGAGTCCATTGATTTGCTTC	
CASP7	F: TAACGACTGCTCTTGTGCCA	141
(XM_604643)	R: GCTGTCTTGCCATCTGTTCC	

Internal control		
H2AFZ	F: AGAGCCGGTTTGCAGTTCCCG	116
(NM_174809)	R: TACTCCAGGATGGCTGCGCTGT	

F: Forward, R: Reverse

Table2. Up- and Down-regulated genes by MK-4 treatment.

a) Up-regulated genes

Ensembl ID	log2 Fold Change	Adj.Pval	Symbol
ENSBTAG00000017007	2.461114166	1.79E-04	TRIB3 (tribbles pseudokinase 3)
ENSBTAG00000014921	2.449137992	2.29E-03	IL6 (Interleukin-6)
ENSBTAG00000000436	2.184366524	4.10E-02	TNFAIP3 (TNF alpha induced protein 3)
ENSBTAG00000018984	2.159385069	1.23E-03	PIK3CD (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta)
ENSBTAG00000005947	2.112214916	2.29E-03	PLAU (plasminogen activator, urokinase)
ENSBTAG00000014972	2.095797253	7.11E-03	PTGER4 (prostaglandin E receptor 4)
ENSBTAG00000031544	2.065043909	3.12E-04	DDIT3 (DNA damage inducible transcript 3)
ENSBTAG00000000396	1.894514299	9.69E-03	PIM1 (Pim-1 proto-onco, serine/threonine kinase)
ENSBTAG00000001164	1.771105294	2.66E-02	ACAD10 (acyl-CoA dehydrogenase family, member 10)
ENSBTAG00000010371	1.723474969	4.42E-05	CHAC1 (ChaC glutathione specific gamma-glutamylcyclotransferase 1)
ENSBTAG00000015618	1.709271336	2.66E-02	GDF15 (Growth differentiation factor 15)
ENSBTAG00000001294	1.696425758	2.80E-04	PPP1R15A (protein phosphatase 1 regulatory subunit 15A)
ENSBTAG00000018517	1.628181331	2.51E-02	VLDLR (very low density lipoprotein receptor)
ENSBTAG00000003721	1.585932683	4.63E-02	CHST1 (carbohydrate sulfotransferase 1)
ENSBTAG00000006016	1.580148876	3.35E-07	GTPBP2 (GTP binding protein 2)
ENSBTAG00000049724	1.5272723	4.10E-02	
ENSBTAG00000052132	1.514267909	3.62E-02	FOXQ1 (forkhead box Q1)
ENSBTAG00000016589	1.476011022	3.10E-02	PRPF40B (pre-mRNA processing factor 40 homolog B)
ENSBTAG00000007718	1.415412002	2.66E-02	TGIF1 (TGFB induced factor homeobox 1)
ENSBTAG00000015591	1.414794722	1.60E-05	SQSTM1 (sequestosome 1)
ENSBTAG00000008197	1.385601005	4.86E-04	EPOR (erythropoietin receptor)
ENSBTAG00000003495	1.318811724	4.73E-02	KDM7A (lysine demethylase 7A)

ENSBTAG00000021435	1.309982877	1.23E-03	MAFF (MAF bZIP transcription factor F)
ENSBTAG00000020283	1.293512886	4.10E-02	DUSP5 (dual specificity phosphatase 5)
ENSBTAG00000013240	1.251476504	4.08E-02	SLC3A2 (solute carrier family 3 member 2)
ENSBTAG00000005339	1.245961794	2.51E-02	VEGFA (vascular endothelial growth factor A)
ENSBTAG00000002395	1.177123204	1.17E-02	HPS3 (HPS3 biosis of lysosomal organelles complex 2 subunit 1)
ENSBTAG00000016010	1.155584116	2.66E-02	KLHL7 (kelch like family member 7)
ENSBTAG00000013860	1.152909703	3.97E-02	GADD45A (Growth arrest and DNA damage inducible alpha)
ENSBTAG00000003245	1.147468004	3.06E-02	BCAR3 (BCAR3 adaptor protein, NSP family member)
ENSBTAG00000006367	1.110662169	3.87E-02	CCN2 (cellular communication network factor 2)
ENSBTAG00000006082	1.109992873	2.66E-02	DNASE2 (deoxyribonuclease 2, lysosomal)
ENSBTAG00000017877	1.102189224	1.06E-02	NECTIN4 (nectin cell adhesion molecule 4)
ENSBTAG00000009863	1.053959663	3.28E-02	BHLHE40 (basic helix-loop-helix family member e40)
ENSBTAG00000025434	1.051051791	1.91E-04	ZFP36L1 (ZFP36 ring finger protein like 1)
ENSBTAG00000023963	1.04080726	3.16E-02	RHBDD1 (rhomboid domain containing 1)
ENSBTAG00000011934	1.035375901	2.51E-02	PCK2 (phosphoenolpyruvate carboxykinase 2, mitochondrial)
ENSBTAG00000015013	1.034400723	2.66E-02	C2CD2L (C2CD2 like)
ENSBTAG00000024539	1.032965721	1.10E-02	SPSB1 (splA/ryanodine receptor domain and SOCS box containing 1)
ENSBTAG00000002922	1.028178649	1.55E-02	GAB2 (GRB2 associated binding protein 2)
ENSBTAG00000013235	1.013137709	2.66E-02	TINAGL1 (tubulointerstitial nephritis antigen like 1)
ENSBTAG00000002069	1.004799324	2.66E-02	BOLA (MHC class I heavy chain)

Adj.Pval; adjusted p-value, calculated by iDEP.

b) Down-regulated genes

Ensembl ID	log2 Fold Change	Adj.Pval	Symbol
ENSBTAG00000010384	-1.51589	2.01E-02	CDC6 (cell division cycle 6)
ENSBTAG00000002719	-1.44119	4.10E-02	ORC1 (origin recognition complex subunit 1)

ENSBTAG00000008216	-1.33388	1.19E-03	RRM2 (ribonucleotide reductase regulatory subunit M2)
ENSBTAG00000014435	-1.25222	2.51E-02	TCF19 (transcription factor 19)
ENSBTAG00000000064	-1.17745	3.06E-02	FEN1 (flap structure-specific endonuclease 1)
ENSBTAG00000003323	-1.132	3.87E-02	NOL6 (nucleolar protein 6)
ENSBTAG00000000532	-1.12283	2.66E-02	EXO5 (exonuclease 5)
ENSBTAG00000039462	-1.07962	4.10E-02	PCLAF (PCNA clamp associated factor)

Adj.Pval; adjusted p-value, calculated by iDEP.

Table3. Enriched GO terms in up-regulated genes

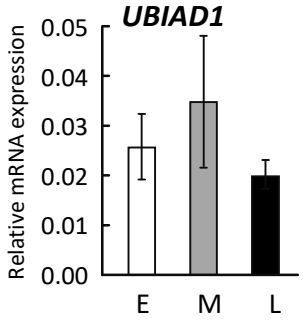
Adj.Pval	nGenens	Pathways
0.000442327	17	Response to organic substance
0.000749619	15	Cellular response to organic substance
0.00098764	13	Apoptotic process
0.001073462	13	Programmed cell death
0.001912089	13	Cell death
0.002102655	22	Developmental process
0.002102655	17	Response to chemical
0.002102655	15	Cellular response to chemical stimulus
0.002102655	6	Response to peptide
0.002102655	10	Response to oxygen-containing compound
0.002102655	9	Cellular response to oxygen-containing compound
0.002125202	11	Positive regulation of cell communication
0.002125202	11	Positive regulation of signaling
0.002125202	17	Cell differentiation
0.002125202	6	Positive regulation of MAPK cascade

dj.Pval; adjusted p-value, calculated by iDEP.

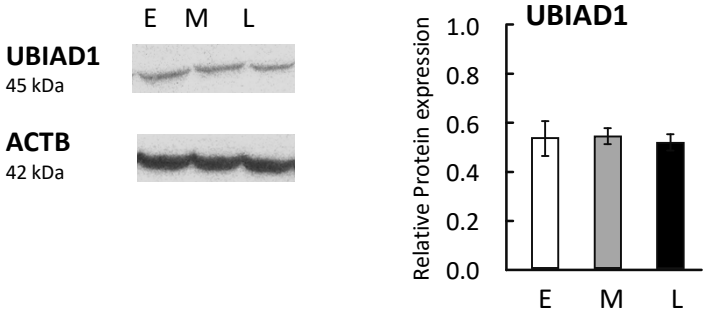
nGenes: Number of genes in the pathway.

Figure 1.

A


UBIAD1
Relative mRNA expression
0.05
0.04
0.03
0.02
0.01
0.00
E
M
L

B


E M L
UBIAD1
45 kDa
ACTB
42 kDa
UBIAD1
Relative Protein expression
1.0
0.8
0.6
0.4
0.2
0.0
E
M
L

C

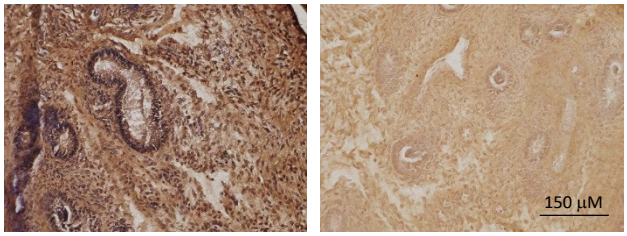

150 µM

Figure 2.

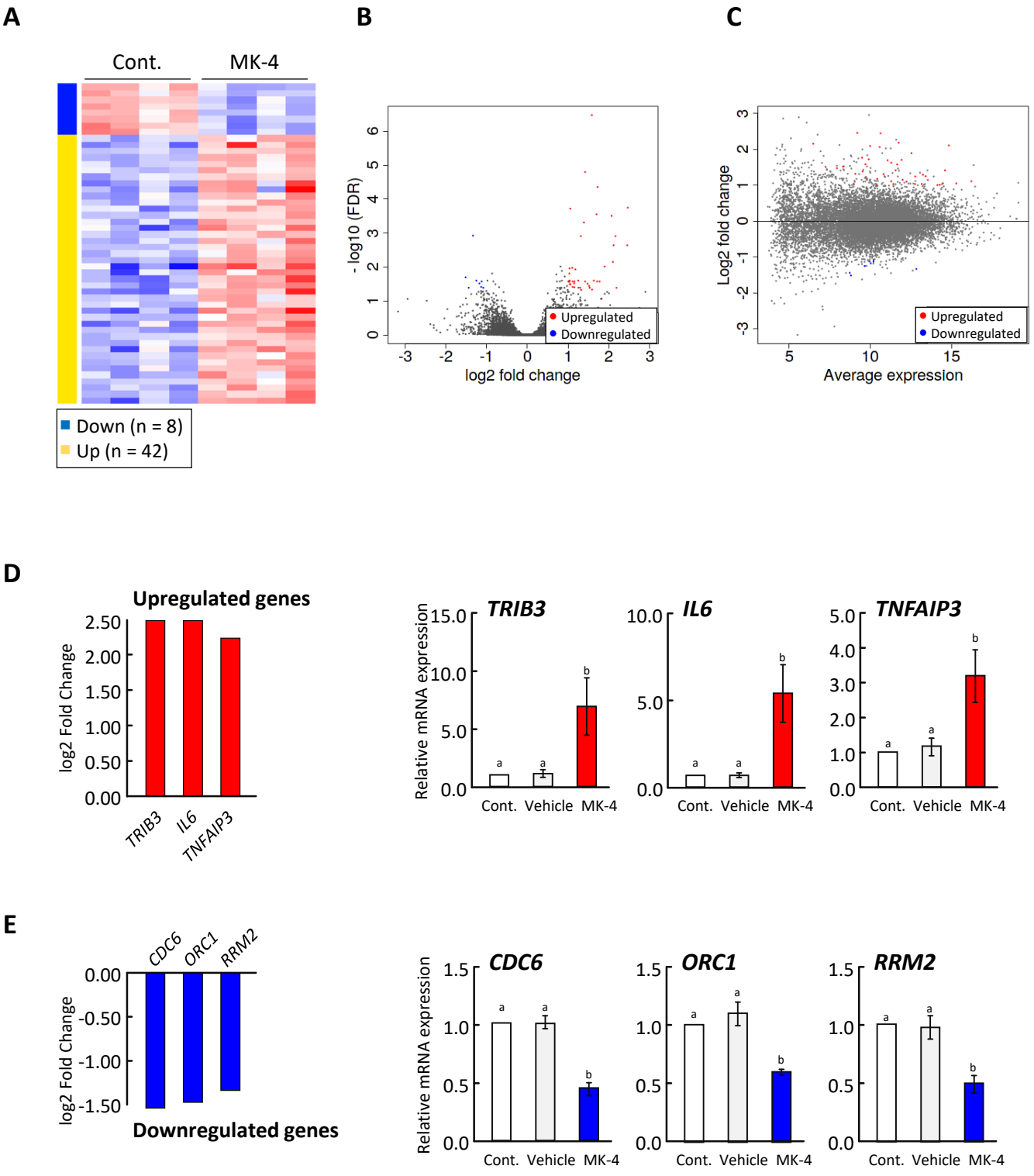
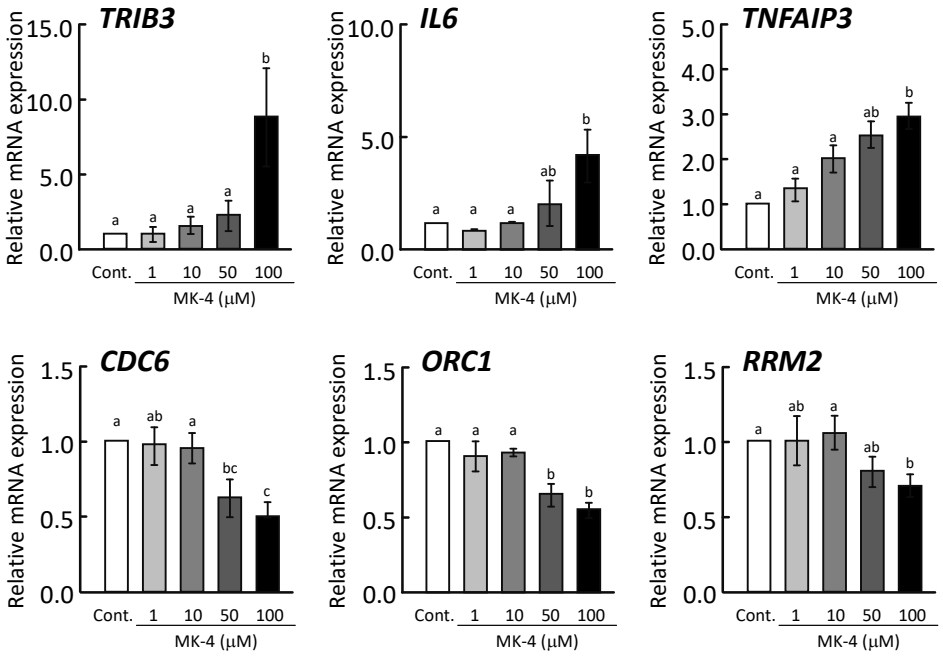

A
Cont.
MK-4
Down (n = 8)
Up (n = 42)
B
- log10 (FDR)
log2 fold change
Upregulated
Downregulated
C
Log2 fold change
Average expression
Upregulated
Downregulated
D
Upregulated genes
log2 Fold Change
TRIB3
IL6
TNFAIP3
Relative mRNA expression
Cont.
Vehicle
MK-4
E
CDC6
ORC1
RRM2
log2 Fold Change
Downregulated genes
Relative mRNA expression
Cont.
Vehicle
MK-4

Figure 3.

A


TRIB3
IL6
TNFAIP3
CDC6
ORC1
RRM2
Relative mRNA expression
Cont.
1
10
50
100
MK-4 (μM)

B

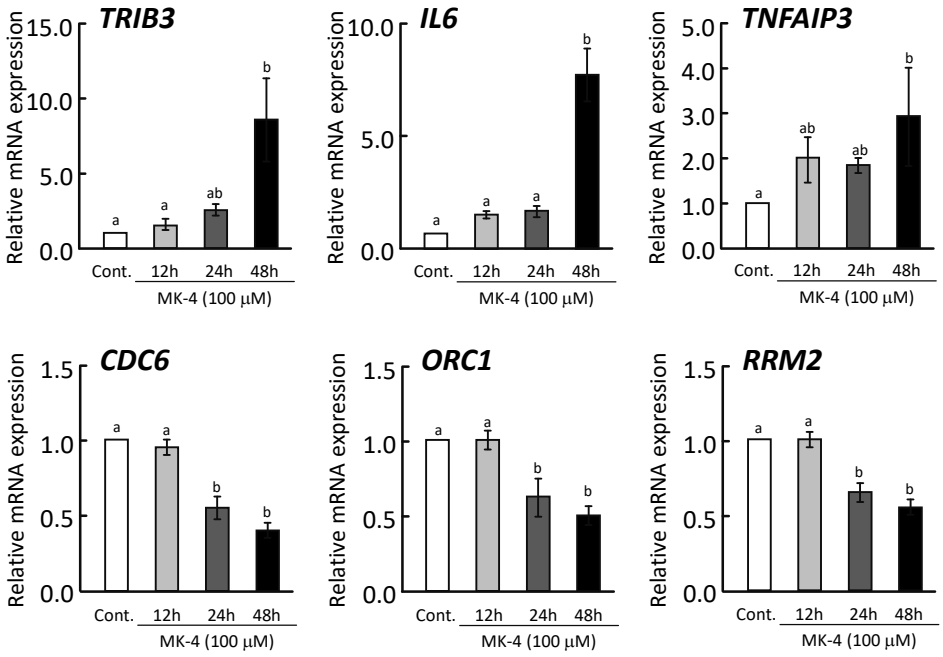
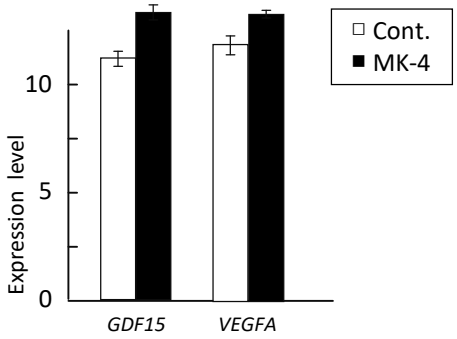
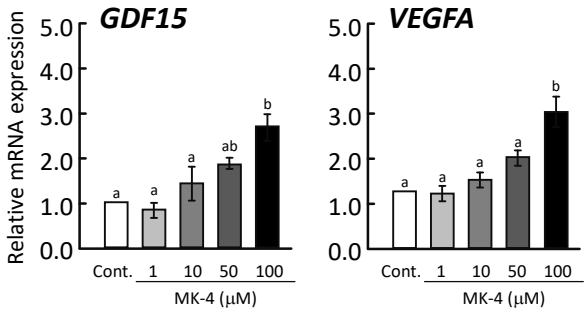

TRIB3
IL6
TNFAIP3
CDC6
ORC1
RRM2
Relative mRNA expression
Cont.
12h
24h
48h
MK-4 (100 μM)

Figure 4.

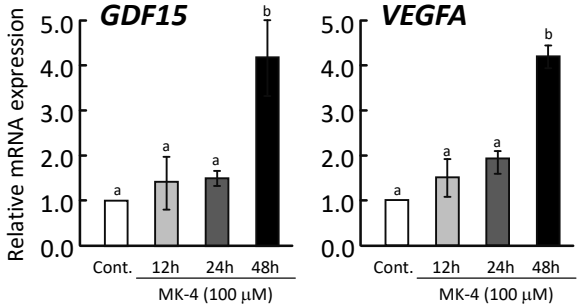
A



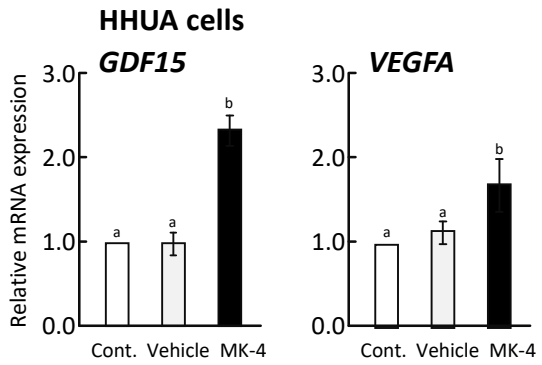
B



C



D



E