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Title	The role of signaling pathways on proliferation and self-renewal of cultured bovine primitive germ cells
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1 **Title: The role of signaling pathways on proliferation and self-renewal of bovine primitive germ**

2 **cells in culture.**

3

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Abstract

Purpose Gonocytes are primitive male germ cells residing in the neonatal testes and are unipotent in nature, but also have a pluripotent stem cell ability in mice under appropriate culture conditions. This study was performed to elucidate molecular mechanisms on self-renewal and survival of bovine gonocytes in culture.

Methods Gonocytes were isolated from neonatal bull calves and were culture in DMEM/F12, supplemented with 15% Knock-out serum replacement (KSR) and growth factor glial cell-derived neurotrophic factor (GDNF). Cells were analyzed at 6 days after culture for cell signaling molecular markers.

Results Colony formation was observed 3-4 days after culture. The addition of GDNF enhanced mitogen-activated protein kinase 1/2 (MAPK1/2) phosphorylation and activated the MAPK signaling pathway. The inhibition of MAPK signaling reduced cell proliferation and abolished colony formation. However, the inhibition of phosphoinositide 3-kinase-AKT (PI3K-AKT) signaling, a dominant pathway for self-renewal of mouse germ cells, did not show any effects on cultured bovine gonocytes. The expression of cell cycle-related regulators cyclin D2 and CDK2 (cyclin-dependent kinase 2) was downregulated with the inhibition of MAPK signaling.

Conclusions These results indicate that the activation of MAPK plays a critical role in the self-renewal and survival of bovine gonocytes via cyclin D1 and CDK2.

Key words: Cell cycle regulators · Gonocytes · MAPK · Signaling Pathways · Self-renewal · Testis

Introduction

Gonocytes reside mostly in the center of the seminiferous tubules and remain quiescent [1]. These cells resume proliferation, migrate to the basement membrane and are transformed to spermatogonial stem cells (SSCs) after arriving at a stem cell niche. The niche referred as specialized microenvironments, which provide architectural support, stimulate to secrete growth factors, and provide extrinsic signals to synchronize self-renewal and differentiation [2].

Understanding the niche factor that regulates germ cell function in rodents has been greatly aided by transplantation assays to immunodeficient mice and the development of a long-term culture system [3]. Culture conditions that support the long-term self-renewal and maintenance of pluripotency of germ cells have been established in various species including mice [4, 5, 6] rats [7], hamsters [8], and rabbits [9]. Growth factor GDNF was shown to be the critical factor for the self-renewal of cultured germ cells in these culture systems. Global gene expression profiling has been identified several intrinsic downstream targets for the GDNF-mediated self-renewal of cultured germ cells. Among these targets, Ets variant 5 (Erm), B cell/lymphoma 6 membrane B (Bcl6b), and LIM homeobox1 (Lhx1) have been identified as core transcription factors associated with the self-renewal of cultured mouse germ cells [10].

The combined approach of RNAi inhibition, microarray analysis, and transplantation assays has revealed the cascade of self-renewal and pluripotency in cultured germ cells. The *ETV5-Bcl6b-Lhx1* cascade under the influence of GDNF was shown to be responsible for the self-renewal and maintenance of mouse germ cells [11]. This mechanism differs from those of mouse ES cells and human ES cells, in which self-renewal and pluripotency maintain the *Oct4-Sox2-Nanog* network [12]. However, the extrinsic signaling pathways for self-renewal and pluripotency respond differently in mice and human ES cells. Instead of different growth factor requirements, common signaling pathways play opposite roles in mice and humans; for example, MAPK inactivation is required for self-renewal in mouse ES cells, while it induces differentiation in human ES cells [13]. Studies on extrinsic signaling pathways of germ cell cultures in mice using a kinase–specific inhibitor demonstrated that PI3K-AKT signaling [14, 15, 16] and Ras-mediated MAPK signaling [17, 18] were

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83 involved in the self-renewal and survival of germ cells. Crosstalk between PI3K/AKT and MAPK

84 signaling was also shown to be essential for the self-renewal of cultured mouse germ cells [15].

85   In domestic species, gene targeting has a potential application in both agriculture and human

86 disease modeling. A combination of gene targeting and pluripotent germ cell lines will provide a

87 time-saving and cost-effective tool for maximizing genetic gain and preserving desirable genetics for

88 the production of superior food animals [19]. The major hindrance in the practical application of this

89 research is the lack of a long-term culture system supporting the self-renewal of germ cells in

90 domestic species. Although germ cells from many mammalian species have been shown to proliferate

91 for more than six months in the seminiferous tubules of immunodeficient mice [20], no germ cell line

92 has been established in livestock species. A possible reason for this is the dearth of understanding on

93 species-specific requirements of growth factors and mechanisms supporting the self-renewal of

94 cultured germ cells.

95   In the present study, we focused on exploring the molecular mechanisms responsible for the

96 self-renewal and maintenance of cultured bovine primitive germ cells (gonocytes). Our results

97 indicated that activation of the MAPK pathway was necessary for the self-renewal and maintenance

98 of cultured bovine gonocytes via the downstream regulation of cyclin D1 and CDK2.

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Materials and methods

Collection of the testes and isolation of gonocytes

102   The testes were collected from 0 to 10-day-old Holstein or Japanese Black bull calves in

103 Dulbecco's modified Eagle's medium and Ham's 12 (DMEM/F12; GIBCOBRL Invitrogen, Carlsbad,

104 CA, USA) supplemented with 15 mM HEPES (Wako Pure Chemical, Tokyo, Japan) from National

105 Livestock Breeding Centre (Fukushima), Gifu Prefectural Livestock Research Institute (Gifu) and

106 Livestock Farm (Kyoto) and were transported to the laboratory on ice within 24 hours.

107   Gonocytes were isolated by a three-step enzymatic digestion method as described previously

108 [21] with minor modifications. Briefly, the testes were decapsulated and minced, and the minced

109 tissues were digested with collagenase Type IV (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) at

110 37 °C for 45 min with constant agitation. After three washes, tissue fragments of the seminiferous

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111   tubules were incubated with collagenase Type IV and hyaluronidase (1 mg/ml; Sigma Aldrich).

112   The cell suspension was further incubated with a mixture of 0.25% trypsin (Nacalai Tesque, Kyoto,

113   Japan) and DNase I (7 mg/ml; Sigma Aldrich) for 10 min. After centrifugation, the resulting pellet

114   was suspended in DMEM/F12 medium containing 10% FBS to stop the enzymatic activity of trypsin.

115   The cell suspension was filtered through a 40 μm nylon mesh (Kyoshin Rikou, Tokyo, Japan) and

116   suspended in DMEM/F12 medium containing 5 % FBS.

117   The cell suspension was subjected to Percoll density gradient (20%-60%) centrifugation at

118   3400 g for 30 min at 21 °C. Cells from the fraction between 35 to 45 % Percoll were separated and

119   plated on 0.2% gelatin-coated dishes (Sigma-Aldrich) for 6 hours in DMEM/F12 medium containing

120   5% FBS. The supernatant containing germ cells was collected and utilized for further experiments.

121   **Cell culture and treatments with cell signaling inhibitors**

122   The culture medium for gonocytes was used DMEM/F12, which was supplemented with 15%

123   Knock-out serum replacement (KSR) (GIBCOBRL, Invitrogen, Carlsbad, CA, USA), 10 μg/ml

124   apotransferrin (Sigma Aldrich), 10 μg/ml insulin (Sigma Aldrich), 110 μg/ml sodium pyruvate

125   (Sigma Aldrich), 0.015% sodium DL-lactate (Sigma Aldrich), NEAA (non-essential amino acid

126   solution, GIBCOBRL Invitrogen, Carlsbad, CA, USA), 100 μM β-mercaptoethanol (Wako Pure

127   Chemical, Tokyo, Japan), 100 μg/ml penicillin (Sigma Aldrich), 50 μg/ml streptomycin (Sigma

128   Aldrich), and 40 μg/ml Gentamycin (Sigma Aldrich) with 1% FBS. GDNF (40 ng/ml, R&D,

129   Minneapolis, MN, USA) was used as a growth factor in this study.

130   Culture dishes were coated with 0.001% poly-L-lysine (P2658, Sigma Aldrich) for 1 hr at

131   37 °C. The dishes were washed once with PBS and utilized for cell culture. Isolated cells were plated

132   at a density of 5×10^5 cells per well of a 6-well dish (Becton Dickinson, Franklin Lakes, NJ, USA) pre-

133   coated with poly-L-lysine and cultured at 37ºC for 6 days in 5% CO_2 in air.

134   Inhibitors of the MEK (PD098059, Stemgent, USA) (PD) and PI3K (LY294002, Cell

135   Signaling, Beverly, MA USA) (LY) signaling pathways were used at a dose of 10 μM [16]. The

136   inhibitor treatment was given on day 3 of culture. Colonies were photographed and counted manually

137   using an inverted microscope (Nikon, DIAPHOT-300, Tokyo, Japan).

138   **Immunofluorescence**

139   Cell smears were prepared on poly-l-lysine-coated glass slides. To stain colonies, cells were

140   cultured for 6 days onto coverslips in 24-well culture dishes (Nunc, DK-4000, Roskilde Denmark).

141   The procedure was performed as described previously (Kim *et al.* 2013). Briefly, cells were fixed in

142   4% paraformaldehyde for 10 min and incubated with 10% goat serum in TBS-T (Tris buffered saline

143   containing 0.1% Triton X-100) for 1 hr at 37 °C. Samples were washed thrice and incubated with

144   primary antibodies at the optimal concentration overnight at 4 °C. The antibodies are used as anti-

145   VASA (1:300; Chemicon, USA) and anti-PGP9.5 (1:200; Biomol, Exeter, Exeter, UK). Samples were

146   again washed thrice and incubated with secondary antibodies such as anti–mouse or anti–rabbit IgG

147   antibodies conjugated with FITC (1:200; DAKO A/S, Denmark) along with DBA-Rhodamine (1:100;

148   Vector Laboratories, Burlingame, USA). The samples were counterstained with DAPI mounting

149   media (Vector Laboratories, Burlingame, CA USA) for 10 min. For negative control, primary

150   antibodies were omitted and the sections was incubated with secondary antibodies Mouse normal IgG,

151   (1:200 dilution, Santa Cruz Biotchnology) and Rabbit normal IgG, (1:200 dilution, Santa Cruze,

152   USA). Photographs were taken with the inverted fluorescent microscope, Eclipse TE2000-U

153   (Olympus BX50, Tokyo, Japan).

154   **RNA isolation and RT-PCR**

155   Total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's

156   protocol. Complementary DNA was synthesized from 1 μg total RNA using ReverTra Ace (MMLV

157   reverse transcriptase RNaseH; Toyobo, Osaka, Japan). To rule out genomic DNA contamination,

158   reactions were performed for samples without the addition of ReverTra Ace (RT-). PCR amplification

159   was performed using 1μL cDNA per 20 μL PCR reaction mixture containing 2 mM MgCl2, 0.25 mM

160   dNTPs, 1× PCR buffer, 5 pmol of each primer, and 1U Taq DNA polymerase (ExTaq; TaKaRa,

161   Tokyo, Japan). Primer sequences are shown in Table 1. PCR products were separated by 1.5%

162   agarose gel electrophoresis and stained with 0.5 μgmL^{-1} ethidium bromide. All PCR products were

163   sequenced to confirm their identity.

164   **Western blot analysis**

165   Isolated cells including gonocytes were cultured for 3 days and were then treated with GDNF,

166   PD and/or LY for 20 min. These cells were lysed in Radioimmunoprecipitation assay buffer (RIPA)

buffer to obtain protein lysates (Abcam, Cambridge, England). Protein concentrations were determined using Coomassie Bradford reagent (Sigma Aldrich). Fifty µg of total protein was mixed with an equal amount of 2x-SDS loading buffer and resolved by SDSPAGE. Electrophoresis was performed using a Mini electrophoresis system (Biocraft, Tokyo, Japan) at 100V for 60 min. The eluted proteins were transferred to an Immobilon-P transfer membrane (Millipore, Massachusetts, USA) at 60V for 90 min. The transmembrane was blocked for nonspecific antibodies with 5% BSA in TBS-T for 90 min at room temperature with gentle shaking. Blots were probed with the primary antibody anti-rabbit pERK (1:5000; Santa Cruz Biotechnology, USA), anti-rabbit p44/42MAPK (1:5000; Cell Signaling, Beverly, MA USA), or anti-mouse α-tubulin (Sigma Aldrich) overnight at 4 °C with gentle shaking. After a brief wash of membranes with TBS-T, membranes were incubated in the secondary antibody ECL-peroxidase labelled anti-rabbit or anti-mouse antibody (1:50000, GE Healthcare, Wisconsin, USA) for 90 min with gentle shaking at room temperature, were washed thrice with TBS-T, and were then developed with an Amersham ECL prime western blotting detection reagent on x-ray film (GE Healthcare, Wisconsin, USA). Density measurements were taken using Imaj J software on scanned x-ray films and normalized using control antibody anti-mouse α-tubulin.

Statistical analysis

All quantification data were presented as the mean ± s.e.m. Analysis of variance (ANOVA) and Turkey's multiple comparison tests were performed using Graph Pad Prism 4.0 (Graph Pad Software, Inc., San Diego CA, USA). Differences were considered to be significant at $P < 0.01$. A densitometric evaluation of western blotting was conducted using Imaj J software with α-tubulin as an internal control.

Results

Gonocytes enrichment and characterization

The enriched gonocytes using Percoll density gradient and differential plating using gelatin-coated dishes were characterized using germ-cell markers DDX4 (Fig. 1A) and PGP9.5 (Fig. 1B).

Effect of the MAPK signaling pathway on self-renewal of cultured germ cells

To investigate the signaling pathways responsible for the self-renewal of gonocytes,

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195 pharmacological inhibitors of the MAPK (PD) and PI3K (LY) signaling pathways were used.

196 Culturing cells in the presence of PD significantly reduced the proliferation of gonocytes and failed to

197 form colonies. However, proliferation and colony formation were not influenced by the presence of

198 LY in the culture (Fig. 2A and B). The appearance of colonies was suppressed in the presence of PD

199 (Fig. 2C).

200 Western blot analysis indicated that the level of MAPK phosphorylation induced in the

201 culture was higher in the presence of GDNF than in the absence of GDNF (Fig. 2D). MAPK

202 phosphorylation was blocked by the addition of PD to the culture medium, but was unaffected by the

203 addition of LY (Fig. 2D).

204 **Enhanced cell cycle regulation of cultured germ cells**

205 The expression patterns of cell cycle regulators in cultured cells treated with signaling inhibitors

206 were analyzed using RT–PCR (Fig. 3A). The addition of GDNF enhanced the expression of cyclin D2

207 and CDK2 (Fig. 3B, C, and F, respectively). The expression of cyclin D1 and CDK2 was significantly

208 reduced by the addition of PD to the culture medium (Fig. 3B and F). However, the enhanced

209 expression of cyclin D2 was significantly reduced by the PD treatment (Fig. 3B). The expression of

210 cyclin D3 was unaffected by the addition of GDNF or PD to the culture medium (Fig. 3D and E).

211 Treatment with the LY inhibitor (PI3K signaling) did not influence the expression of these genes.

212

213 **Discussion**

214 Signaling pathways that regulate the self-renewal and differentiation of germ cells in culture

215 have been well documented in mice [22]. However, the mechanisms of proliferation of cultured germ

216 cells have yet been elucidated in species other than mice. Although several attempts have been made

217 to develop a long-term culture system for bovine gonocytes, colony formation could not obtain after

218 subsequent passages [23, 24]. We previously established a long-term culture system of bovine

219 gonocytes for more than 1.5 months [25]. In this study, culture condition was stable to maintain cell

220 survival and proliferation of bovine gonocytes, mouse embryonic stem (ES)-like colonies appeared in

221 culture and expressed pluripotent marker genes (*OCT3/4* and *NANOG*) [25].

222 GDNF was shown to be a molecule that regulates self-renewal and differentiation of mouse

8

223   SSCs [26]. GDNF signals act though the multicomponent receptor complex comprised of GFRα-1 and

224   RET tyrosin kinases in various cell types [27]. GFRα-1 and RET have also been recognized as

225   spermatogonial markers expressed in gonocytes, SSCs, and differentiated spermatogonia [28]. These

226   co-receptors of GDNF-mediated signaling were shown to be necessary for the self-renewal of germ

227   cells in rodents [29]. The GDNF enhanced cell proliferation and colony formation bovine gonocytes

228   were reported by Aponte et al [30, 31], which indicated that GDNF-mediated signaling was conserved

229   in germ cell cultures in rodents and cattle.

230   In the present study, we showed that the inhibition of MAPK pathways by the inhibitor

231   PD98095 impaired cell proliferation and abolished colony formation (Fig. 2A and 2B). The presence

232   of GDNF significantly increased tyrosine phosphorylation of MAPK44/42 (Fig. 3D and 3E). This

233   stimulation was blocked by the treatment with PD98059 (Fig. 3D and 3E). These results indicate that

234   the activation of MAPK pathways is essential for the self-renewal of bovine gonocytes in culture. In

235   accordance with these results, GDNF signals were previously shown to activate RET phosphorylation

236   and subsequently activate MAPK pathways, which are essential for the cell growth and proliferation

237   of SSCs in mice [17]. Previous studies also demonstrated that FGF2, not GDNF, mediates the

238   activation of the MAPK pathway by upregulating the downstream targets ETV5 and Bcl6b in mouse

239   germ cell culture [32]. However, the addition of FGF2 to our culture system enhanced somatic cell

240   proliferation and induced the differentiation of gonocytes (unpublished data).

241   PI3K/AKT is known to play an important role in the self-renewal of germ cells in mice through

242   GDNF or FGF2 stimulation [33]. The activation of PI3K/AKT signaling in mouse germ cells was

243   shown to be completely inhibited by the inhibitor LY294002, which impaired the self-renewal of

244   cultured germ cells [15, 16]. However, the activation of AKT alone was not sufficient for the self-

245   renewal of SSCs [15]. Src kinase is an alternative activator of PI3K pathways, which results in the

246   upregulation of *N-myc* expression and promotes the proliferation and self-renewal of mouse germ

247   cells [14, 16]. Our results showed that the inhibition of PI3K/AKT signaling by LY294002 did not

248   affect the cell proliferation or colony formation of bovine germ cells. This result indicated that AKT-

249   or Src-mediated PI3K signaling did not play a significant role in the self-renewal of bovine gonocytes

250   in culture. This finding is in contrast to that reported in mice, in which PI3K was shown to be the

251   dominant signaling pathway.

252   The inhibition of MAPK and PI3K signaling was previously shown to result in the

253   downregulation of pluripotency genes *OCT3/4*, *NANOG,* and *SOX2* in human ES cell lines [34, 35],

254   which indicated that these signaling pathways play essential roles in maintaining the self-renewal and

255   pluripotency of human ES cells. PI3K/AKT signaling was also shown to regulate expression of the

256   self-renewal cascade genes *Bcl6b, Etv5, and Lhx1* germ cell culture in mice [16]. Interestingly, the

257   expression of *Oct3/4* was essential for the survival of mouse germ cells, but was not influenced by

258   GDNF and did not play a significant role in self-renewal [36]. However, the expression of *OCT3/4*

259   and *NANOG* was detected in bovine gonocytes in culture [25] and gonocytes in the testes of pigs [37]

260   and cattle [25], suggesting that these pluripotent genes have roles in the maintenance and self-renewal

261   of gonocytes in domestic species. In contrast, *Nanog* expression has not been detected in cultured

262   bovine gonocytes or in the testes of mice [38]. Our previous report [25] demonstrated that the strong

263   expression of the pluripotency markers *OCT3/4* and *NANOG* in cultured bovine germ cells was

264   associated with the appearance of mouse ES-like colonies. These results indicate that the different

265   expressions of transcription factors in mice and domestic species may lead to different regulatory

266   mechanisms for the self-renewal and colony formation of cultured germ cells. However, the role of

267   these genes has to be elucidated further to understand the MAPK-mediated self-renewal of bovine

268   gonocytes germ cells.

269   Activation of the extrinsic MAPK [17, 18] and PI3K [15] signaling pathways in mouse germ

270   cells was previously shown to be involved in the regulation of cell-cycle-related cyclin gene

271   expressions. To understand the relationship between signaling pathways and the self-renewal of

272   bovine gonocytes in culture, we analyzed the downstream genes potentially involved in cell cycle

273   regulation. Cyclin D1 is essential for the entry to the G1/S-phase of the cell cycle in the presence or

274   absence of GDNF and is regulated by the MAPK pathway [39]. The expression of cyclin D1 has also

275   been observed in proliferating germ cells and SSCs in the mouse testes [40]. In this study, the

276   expression of cyclin D1 was significantly downregulated after the inhibition of MAPK signaling by

277   PD (Fig. 3A and 3B), but was unaffected by the presence of GDNF. In contrast, cyclin D2 expression

278   was significantly upregulated upon GDNF stimulation and inhibited upon pre-treatment with the

279   MAPK inhibitor (Fig. 3A and 3C), which indicated that the MAPK pathway was involved in

280   regulating the cell cycle of bovine gonocytes. The overexpression of cyclin D2 was previously shown

281   to regulate the self-renewal of germ cells and was mediated by Ras activation in mice [18]. CDK2 has

282   been shown to be involved in controlling the entry to the S-phase in association with cyclin A. CDK2

283   was upregulated in the presence of GDNF and controlled entry to the G1/S-phase of mouse C18-4

284   germ cell lines via MAPK-mediated signaling [17]. In this study, CDK2 expression was also

285   significantly upregulated upon GDNF stimulation and the inhibition of MAPK signaling resulted in

286   the downregulation of CDK2 expression (Fig. 3A and 3E). Enhanced CDK kinase activity was

287   previously shown to be essential for the Ras-induced proliferation of cultured mouse germ cells [18].

288   Our results suggested that cell cycle-related genes were not influenced by the inhibition of PI3K

289   signaling. This is consistent with a previous report [16], in which the inhibition of PI3K signaling

290   does not significantly affect changes in cyclin gene expression in mouse germ cells in culture.

291   Taken together, these findings reveal the unique and crucial role of MAPK signaling in

292   maintaining the self-renewal and colony formation of bovine gonocytes in culture. In contrast to our

293   findings, cultured mouse germ cells require the crosstalk between MAPK and PI3K signaling

294   pathways for self-renewal. The downstream targets of MAPK signaling that ultimately influence the

295   self-renewal of bovine gonocytes need to be determined in future experiments. The present study has

296   revealed the marked differences in the control of the self-renewal and survival of cultured germ cells

297   in mice and cattle. These results will be useful for identifying optimal culture conditions to establish a

298   long-term culture system and germ-cell lines in domestic species.

299

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307

308 **Disclosures**

309 **Conflict of interest:** Mahesh Sahare, Ayagi Otomo, Kana Komatsu, Naojiro Minami, Masayasu

310 Yamada, and Hiroshi Imai declare that they have no conflict of interest.

311 **Animal studies:** All institutional and national guidelines for the care and use of animals were

312 followed.

313 **Human rights:** This article does not contain any studies with human subjects performed by any of the

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315

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411

Figure Legends

414 **Figure 1.** Immunocytochemical characterization of cultured gonocytes in the presence of GDNF by

415 using germ cell-specific antibodies (DDX4 and PGP9.5). A) DDX4 expression with the nuclear

416 marker DAPI, B) PGP9.5 expression with DAPI, E) anti-rabbit IgG as a control (Magnification=

417 40X).

418

419 **Figure 2.** Effects of MAPK and PI3K signaling inhibitors on the self-renewal and colony formation

420 of cultured gonocytes. A) Cell proliferation of cultured gonocytes for 6 days in the presence of MAPK

421 (PD) and PI3K (LY) inhibitors. Cell proliferation was significantly inhibited in the presence of PD

422 relative to that in the absence of GDNF as a control (GD-), in the presence of GDNF (GD+) or LY.

423 *$P< 0.01$ and **$P< 0.01$ significantly different from GD-, respectively; [####]$P< 0.01$ significantly

424 different from GD+. (Data were collected n=3 in each experiment, from three independent

425 experiments, and indicated as the mean ± s.e.m). B) Colony formation by cultured gonocytes for 6

426 days in the presence of MAPK/PI3K inhibitors. The number of colonies formed was significantly less

427 in the PD-treated group than in the GD- group. The number of colonies formed was higher in the GD+

428 culture than in the GD- culture. ****$P< 0.01$, ***$P< 0.01$ and **$P< 0.01$ significantly different from

429 GD-; [####]$P< 0.01$ significantly different from GD+. (Data were collected n=3 in each experiment,

430 from three independent experiments, and indicated as the mean ± s.e.m). C) Appearance of colonies in

431 the control (GD-), and in the presence of GD+, PD, and LY (Magnification= 100X). D) Western blot

432 analysis of MAPK and phosphorylated MAPK (pMAPK). (Gonocytes were cultured for 4 days in the

433 presence of GDNF. As a control, cells were starved for 16 hours without GDNF and treated with no

434 chemicals, PD and LY for 20 min. E) Estimation of phosphorylated MAPK expression based on

435 western blot from three independent immunoblot experiments (mean ± s. e. m). The level of

436 phosphorylated MAPK was significantly lower in PD-treated cells than in GD+ treated cells (**$P<$

437 0.01). However, the level of phosphorylated MAPK was not significantly different in the absence of

438 GD (GD-) and in the presence of LY.

439

Figure 3. Effect of the inhibition of MAPK and PI3K signaling on the expression of cell cycle

regulator genes. A) RT-PCR analysis of cell cycle regulator genes (cyclin D1, cyclin D2, cyclin D3

and CDK2) and b-Actin as the housekeeping gene. Relative mRNA expression of cyclin D1 (B),

cyclin D2 (C), cyclin D3 (D), and CDK2 (E) were examined in the presence of GDF or MAPK/PI3K

inhibitors. Data represented from three independent gel images (mean ± s.e.m). *P< 0.01 and **P<

0.01 significantly different from GD-, [#]P< 0.01 and [##]P< 0.01 significantly different from GD+.

468
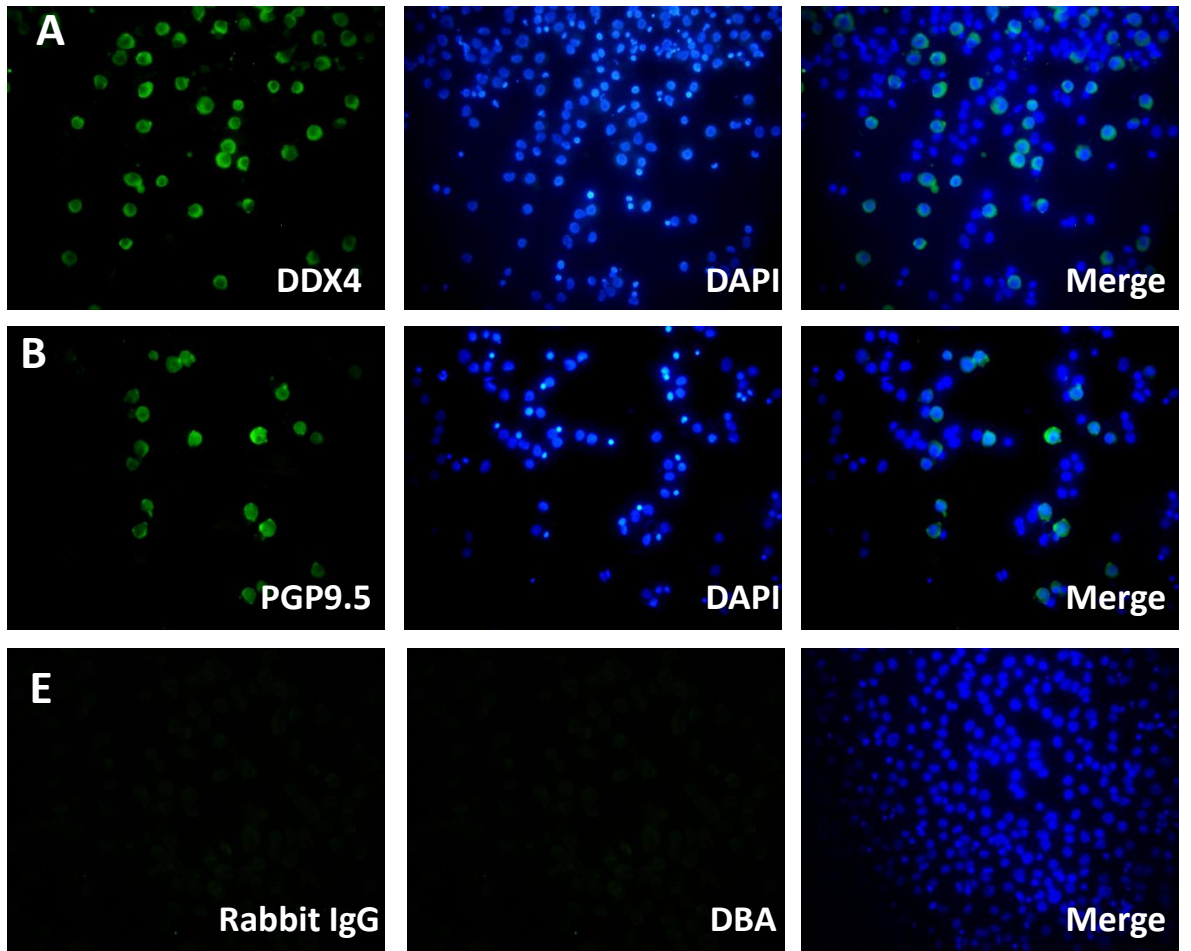
Figure 1.

Figure 2.

A

B

Figure 2.

C

GD-	GD+
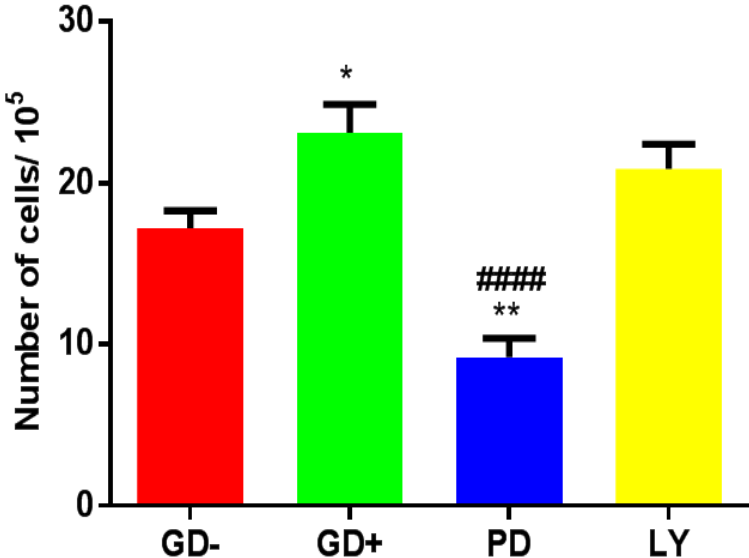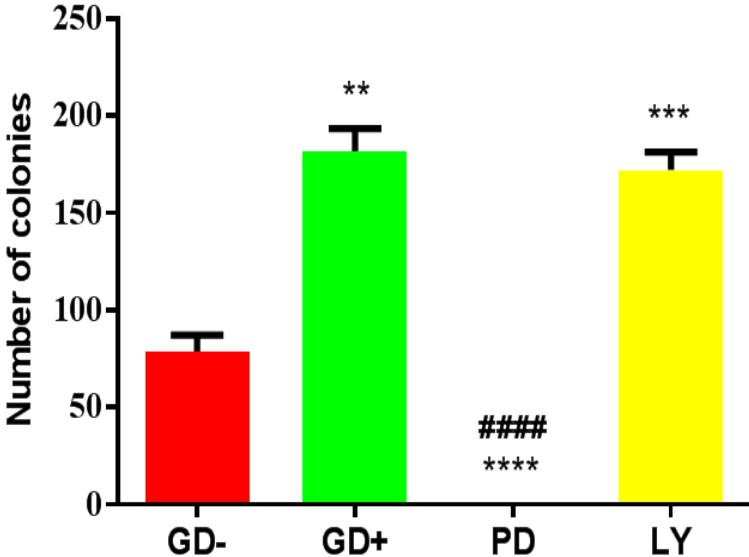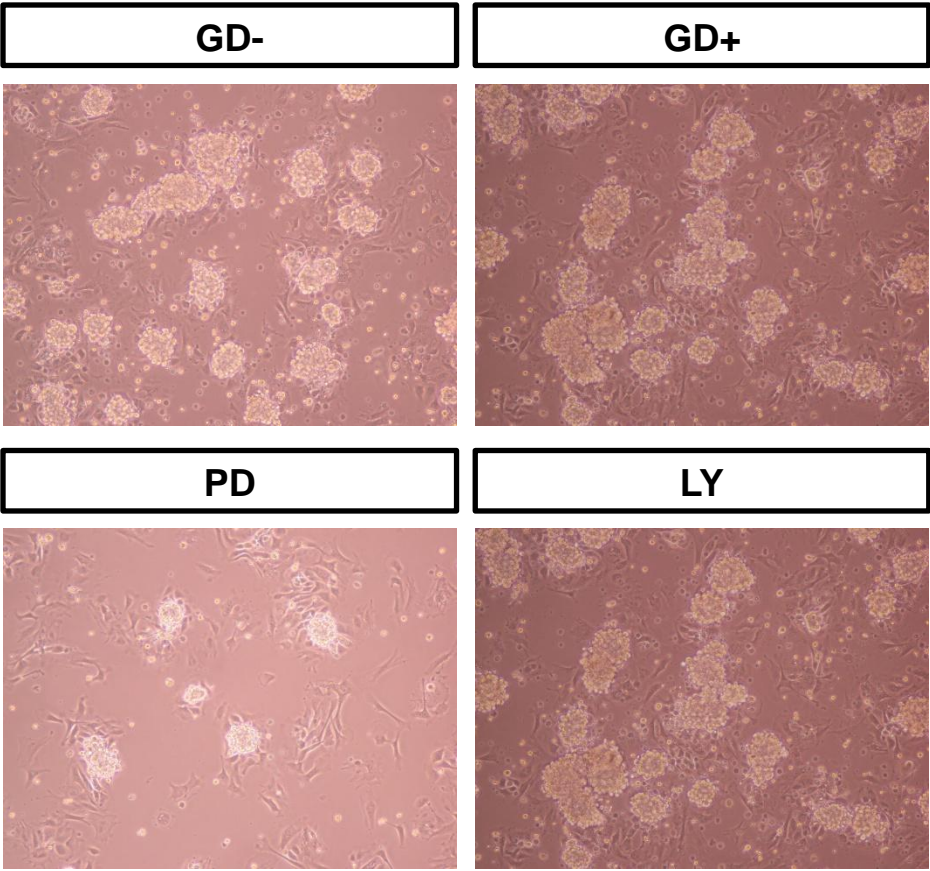
PD	LY

D

MAPK44/42

pMAPK44/42

b- actin

GD	-	+	+	+
PD	-	-	+	-
LY	-	-	-	+

E

Figure 3.
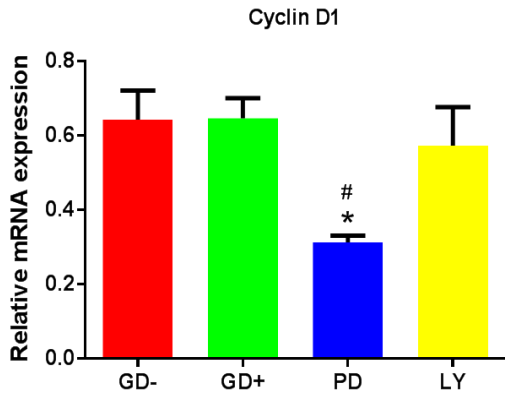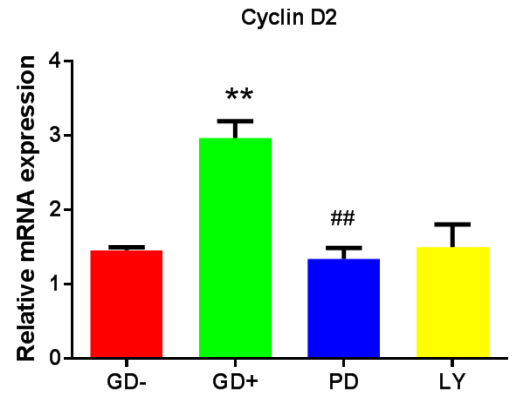
A

B

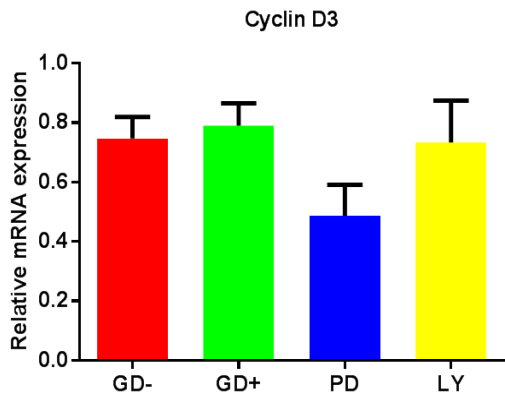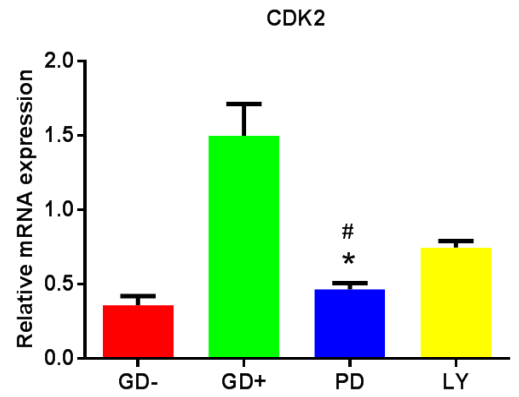Cyclin D1

C

Cyclin D2

D

Cyclin D3

E

CDK2
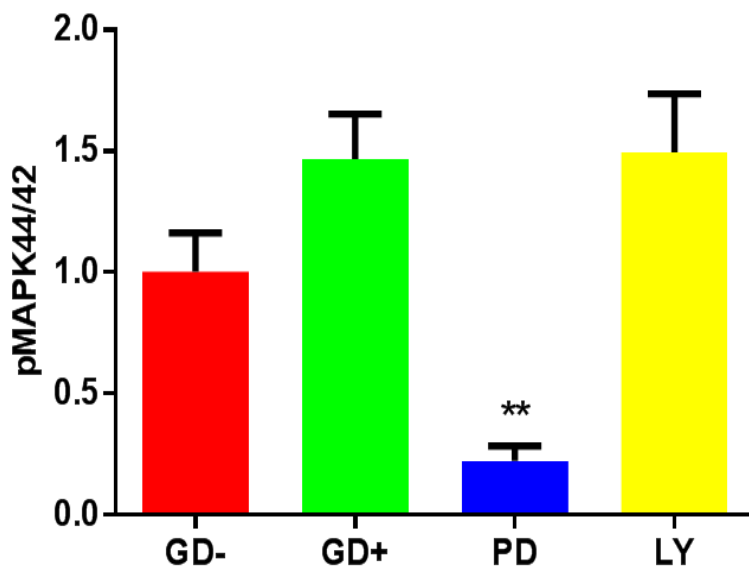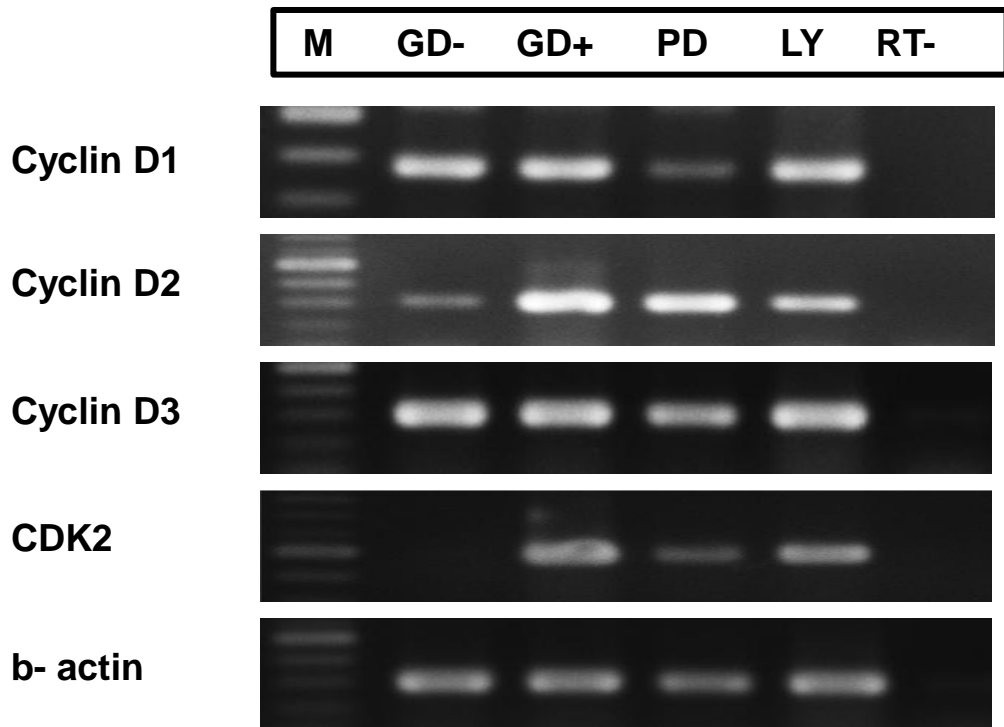
Table 1. RT-PCR primer sequences used for the amplification of specific genes

Gene Name	Primer sequence (5'-3')	Product Size (bp)	Accession no.
Cyclin D1	F: GCCGAGGAGAACAAGCAGAT	378	NM_001046273.2
	R:TCAGATGTTCACGTCACGCA		
Cyclin D2	F: GCAGAACTTGCTGACCATCG	319	NM_001034709.2
	R: AGGCTTGATGGAGTTGTCGG		
Cyclin D3	F: CACTTGGAGGCCCTGCATAA	495	NM_001034709.2
	R: GGTAGCATGATGGTCCTCGG		
CDK2	F: GGGAACGTACGGAGTTGTGT	491	NM_001014934.1
	R: CCAGAAGGATTTCCGGTGCT		
b-Actin	F: CGATCCACACAGAGTACTTGCG	316	NM_173979.3
	R: CGAGCGTGGCTACAGTTCACC		