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ORIGINAL ARTICLE

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

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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 pluripotent stem cell ability in mice under appropriate culture conditions. This study was performed to elucidate the molecular mechanisms of self-renewal and survival of cultured bovine gonocytes.
Methods Gonocytes were isolated from neonatal bull calves and were cultured in DMEM/F12 supplemented with 15 % knock-out serum replacement (KSR) and glial cell-derived neurotrophic factor (GDNF). Cells were analyzed six days after culturing for cell-signaling molecular markers.
Results Colony formation was observed 3–4 days after being cultured. Addition of GDNF enhanced mitogen-activated protein kinase 1/2 (MAPK1/2) phosphorylation and activated the MAPK signaling pathway. Inhibition of MAPK signaling reduced cell proliferation and abolished colony formation. However, 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. Expression of cell cycle-related regulators cyclin D2 and cyclin-dependent kinase 2 (CDK2) was downregulated with inhibition of MAPK signaling.
Conclusions These results indicate activation of MAPK plays a critical role in self-renewal and survival of bovine gonocytes via cyclin D1 and CDK2.

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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 refers to a specialized microenvironment that provides architectural support, stimulates secretion of growth factors and provides extrinsic signals for synchronizing 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 long-term self-renewal and maintenance proliferation of germ cells have been established in various species including mice [4–6], rats [7], hamsters [8] and rabbits [9]. GDNF was shown to be a critical factor for self-renewal of cultured germ cells in these culture systems. Global gene expression profiling has 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 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 [14–16] and Ras-mediated MAPK signaling [17, 18] were involved in self-renewal and survival of germ cells. Cross-talk between PI3K/AKT and MAPK signaling was also shown to be essential for the self-renewal of cultured mouse germ cells [15].

Gene targeting has a potential application in both agriculture and human disease modeling in domesticated species. A combination of gene targeting and pluripotent germ cell lines will provide a time-saving and cost-effective tool for maximizing genetic gain and preserving desirable genetics for the production of superior food animals [19]. The major hindrance in the practical application of this research is the lack of a long-term culture system supporting the self-renewal of germ cells in domesticated species. Although germ cells from many mammalian species have been shown to proliferate for more than six months in the seminiferous tubules of immunodeficient mice [20], no germ cell line has been established in livestock species. A possible reason for this is the dearth of understanding about species-specific requirements of growth factors and mechanisms supporting self-renewal of cultured germ cells.

In the present study we focused on exploring the molecular mechanisms responsible for self-renewal and maintenance of cultured bovine primitive germ cells (gonocytes). Our results indicated that activation of the MAPK pathway was necessary for self-renewal and maintenance of cultured bovine gonocytes via the downstream regulation of cyclin D1 and CDK2.

Materials and methods

Collection of the testes and isolation of gonocytes

Testes were collected from 0- to 10-day old Holstein or Japanese black bull calves in Dulbecco's modified Eagle's medium and Ham's 12 (DMEM/F12; GIBCOBRL Invitrogen, Carlsbad, CA, USA) supplemented with 15 mM HEPES (Wako Pure Chemical, Tokyo, Japan); the bull calves were from the National Livestock Breeding Centre (Fukushima), Gifu Prefectural Livestock Research Institute (Gifu) and the Livestock Farm (Kyoto) and were transported to the laboratory on ice within 24 h.

Gonocytes were isolated by a three-step enzymatic digestion method as described previously [21] with minor modifications. Briefly, the testes were decapsulated and minced and the minced tissue was digested with collagenase Type IV (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 45 min with constant agitation. After three washes tissue fragments of the seminiferous tubules were incubated with collagenase Type IV and hyaluronidase (1 mg/ml; Sigma Aldrich). The cell suspension was further incubated with a mixture of 0.25 % trypsin (Nacalai Tesque, Kyoto, Japan) and DNase I (7 mg/ml; Sigma Aldrich) for 10 min. After centrifugation, the resulting pellet was suspended in DMEM/F12 medium containing 10 % FBS to stop the enzymatic activity of trypsin. The cell suspension was filtered through a 40 μm nylon mesh (Kyoshin Rikou, Tokyo, Japan) and suspended in DMEM/F12 medium containing 5 % FBS.

The cell suspension was subjected to Percoll density gradient (20–60 %) centrifugation at 3,400*g* for 30 min at 21 °C. Cells from the fraction between 35 and 45 % Percoll were separated and plated on 0.2 % gelatin-coated dishes (Sigma-Aldrich) for 6 h in DMEM/F12 medium containing 5 % FBS. The supernatant containing germ cells was collected and utilized for further experiments.

Cell culture and treatments with cell signaling inhibitors

The culture medium for gonocytes was DMEM/F12 supplemented with 15 % knock-out serum replacement (KSR; GIBCOBRL, Invitrogen, Carlsbad, CA, USA), 10 μg/ml apotransferrin (Sigma Aldrich), 10 μg/ml insulin (Sigma Aldrich), 110 μg/ml sodium pyruvate (Sigma Aldrich), 0.015 % sodium DL-lactate (Sigma Aldrich), NEAA (non-essential amino acid solution; GIBCOBRL Invitrogen, Carlsbad, CA, USA), 100 μM β-mercaptoethanol (Wako Pure Chemical, Tokyo, Japan), 100 μg/ml penicillin (Sigma Aldrich), 50 μg/ml streptomycin (Sigma Aldrich) and 40 μg/ml gentamycin (Sigma Aldrich) with 1 % FBS. GDNF (40 ng/ml; R&D, Minneapolis, MN, USA) was used as a growth factor in this study.

Culture dishes were coated with 0.001 % poly-L-lysine (P2658, Sigma Aldrich) for 1 h at 37 °C. The dishes were washed once with PBS and utilized for cell culture. Isolated cells were plated at a density of 5×10^5 cells per well of a 6-well dish (Becton–Dickinson, Franklin Lakes, NJ, USA) pre-coated with poly-L-lysine and cultured at 37 °C for 6 days in 5 % CO_2 in air.

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		
β-Actin	F: CGATCCACACAGAGTACTTGCG	316	NM_173979.3
	R: CGAGCGTGGCTACAGTTCACC		

Inhibitors of the MEK (PD098059, Stemgent, USA) (PD) and PI3K (LY294002, Cell Signaling, Beverly, MA USA) (LY) signaling pathways were used at a dose of 10 μM [16]. The inhibitor treatment was given on day 3. Colonies were photographed and counted manually using an inverted microscope (Nikon, DIAPHOT-300, Tokyo, Japan).

Immunofluorescence

Cell smears were prepared on poly-L-lysine-coated glass slides. To stain colonies cells were cultured for 6 days onto coverslips in 24-well culture dishes (Nunc, DK-4000, Roskilde Denmark). The procedure was performed as described previously [21]. Briefly, cells were fixed in 4 % paraformaldehyde for 10 min and incubated with 10 % goat serum in TBS-T (Tris-buffered saline containing 0.1 % Triton X-100) for 1 h at 37 °C. Samples were washed thrice and incubated with primary antibodies at the optimal concentration overnight at 4 °C. The antibodies are used as anti-DDX4 (1:300; Chemicon, USA) and anti-UCHL-1 (1:200; Biomol, UK). Samples were again washed thrice and incubated with secondary antibodies such as anti-mouse or anti-rabbit IgG antibodies conjugated with FITC (1:200; DAKO A/S, Denmark) along with DBA-Rhodamine (1:100; Vector Laboratories, Burlingame, USA). The samples were counterstained with DAPI mounting media (Vector Laboratories, Burlingame, CA USA) for 10 min. For negative control primary antibodies were omitted and the sections were incubated with secondary antibodies, mouse normal IgG (1:200 dilution; Santa Cruz Biotchnology) and rabbit normal IgG (1:200 dilution; Santa Cruz, CA, USA). Photographs were taken with an inverted fluorescent microscope (Eclipse TE2000-U, Olympus BX50; Tokyo, Japan).

RNA isolation and RT-PCR

Total RNA was isolated with a Trizol reagent (Invitrogen) according to the manufacturer's protocol. Complementary DNA was synthesized from 1 μg total RNA using ReverTra Ace (MMLV reverse transcriptase RNaseH; Toyobo, Osaka, Japan). To rule out genomic DNA contamination reactions were performed for samples without the addition of ReverTra Ace (RT-). PCR amplification was performed using 1μL cDNA per 20 μL PCR reaction mixture containing 2 mM MgCl2, 0.25 mM dNTPs, 1 × PCR buffer, 5 pmol of each primer and 1 U Taq DNA polymerase (ExTaq; TaKaRa, Tokyo, Japan). Primer sequences are shown in Table 1. PCR products were separated by 1.5 % agarose gel electrophoresis and stained with 0.5 μg mL^{-1} ethidium bromide. All PCR products were sequenced to confirm their identity.

Western blot analysis

Isolated cells including gonocytes were cultured for 3 days and were then treated with GDNF, PD and/or LY for 20 min. These cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer to obtain protein lysates (Abcam, Cambridge, England). Protein concentrations were determined using a Coomassie Bradford reagent (Sigma Aldrich). Fifty μg of total protein was mixed with an equal amount of 2x-SDS loading buffer and resolved by SDS-PAGE. Electrophoresis was performed using a mini-electrophoresis system (Biocraft, Tokyo, Japan) at 100 V for 60 min. The eluted proteins were transferred to an Immobilon-P transfer membrane (Millipore, Massachusetts, USA) at 60 V 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, Massachusetts, 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 (1:50,000; GE Healthcare, Wisconsin, USA) for 90 min with gentle shaking at room temperature,

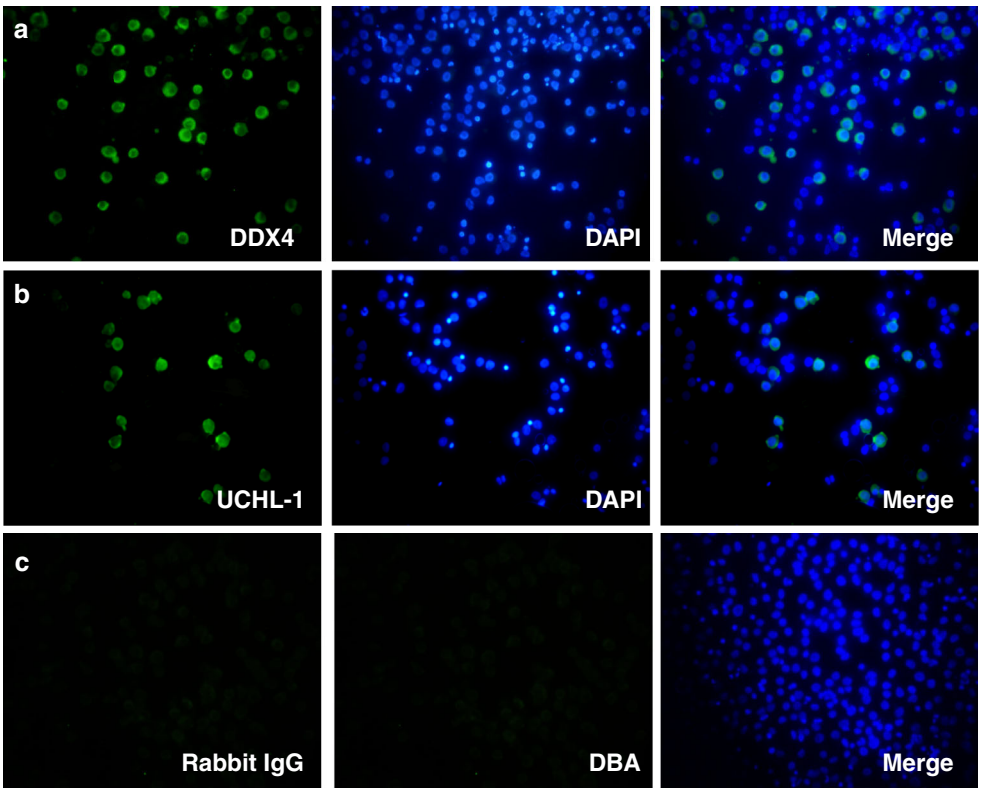

Fig. 1 Immunocytochemical characterization of cultured gonocytes in the presence of GDNF by using germ cell-specific antibodies (DDX4 and UCHL.1). **a** DDX4 expression with the nuclear marker DAPI; **b** UCHL-1expression with DAPI; **c** anti-rabbit IgG as a control (×40)

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

Gonocyte enrichment and characterization

Enriched gonocytes using a Percoll density gradient and differential plating using gelatin-coated dishes were characterized using germ-cell markers DDX4 (Fig. 1a) and UCHL-1 (Fig. 1b). The overall purity of germ cells, assessed by localization of germ cell marker DDX4, was approximately 79.00 ± 2.08 % ($n = 4$) (Fig. 1a).

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

To investigate the signaling pathways responsible for self-renewal of gonocytes pharmacological inhibitors of the MAPK (PD) and PI3K (LY) signaling pathways were used. Culturing cells in the presence of PD significantly reduced the proliferation of gonocytes and failed to form colonies. However, proliferation and colony formation were not influenced by the presence of LY in the culture (Fig. 2a, b). The appearance of colonies was suppressed in the presence of PD (Fig. 2c).

Western blot analysis indicated the level of MAPK phosphorylation induced in the culture was higher in the presence of GDNF than in the absence of GDNF (Fig. 2d). MAPK phosphorylation was blocked by the addition of PD to the culture medium, but was unaffected by the addition of LY (Fig. 2d).

Enhanced cell cycle regulation of cultured germ cells

The expression patterns of cell cycle regulators in cultured cells treated with signaling inhibitors were analyzed using

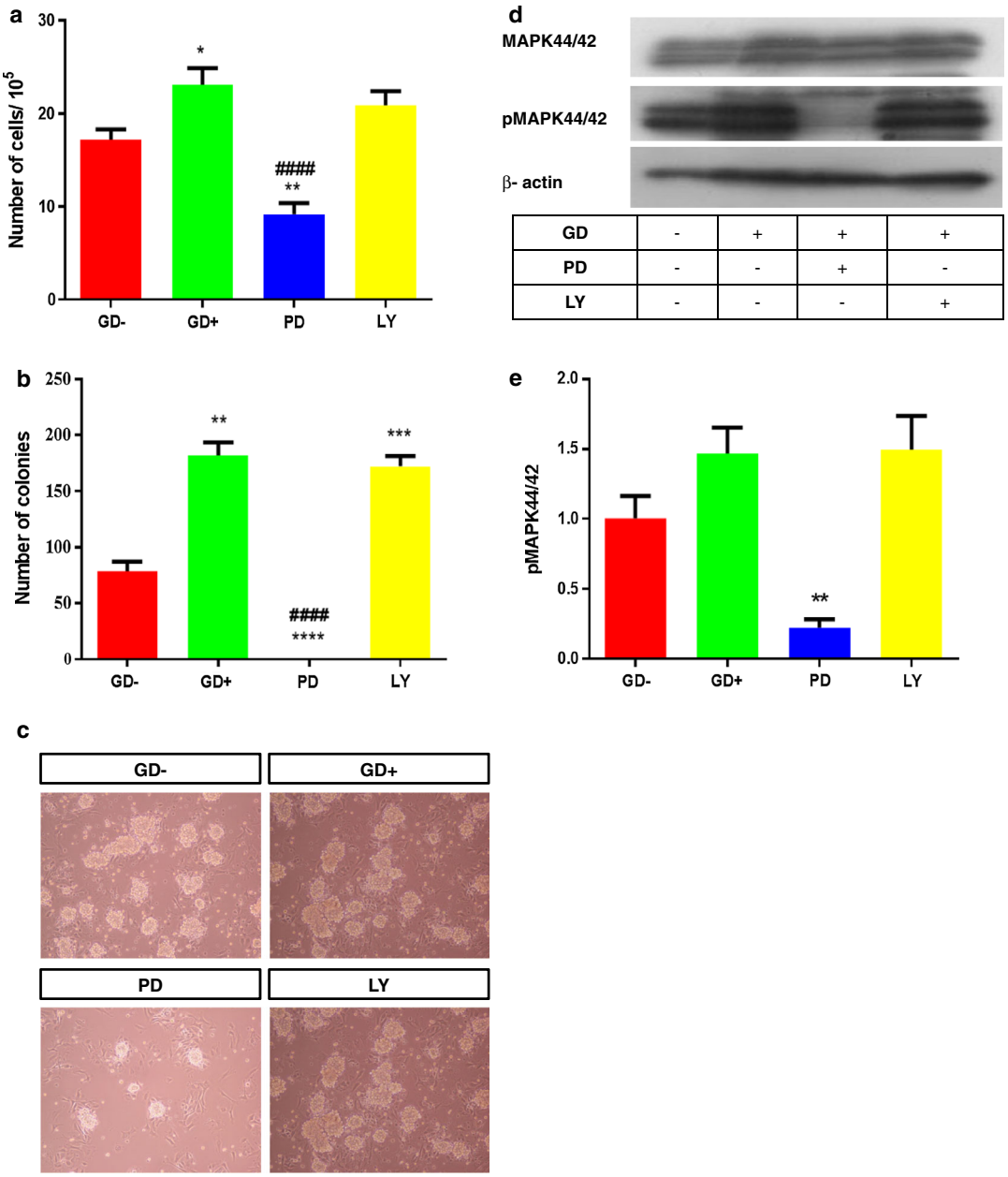

Fig. 2 Effects of MAPK and PI3K signaling inhibitors on the self-renewal and colony formation of cultured gonocytes. **a** Cell proliferation of cultured gonocytes for 6 days in the presence of MAPK (PD) and PI3K (LY) inhibitors. Cell proliferation was significantly inhibited in the presence of PD relative to that in the absence of GDNF as a control (GD−), in the presence of GDNF (GD+) or LY, $^{*}P < 0.01$ and $^{**}P < 0.01$ significantly different from GD−; $^{\#\#\#\#}P < 0.01$ significantly different from GD+. (Data were collected $n = 3$ in each experiment, from three independent experiments and indicated as the mean ± s.e.m); **b** Colony formation by cultured gonocytes for 6 days in the presence of MAPK/PI3K inhibitors. The number of colonies formed was significantly less in the PD-treated group than in the GD− group. The number of colonies formed was higher in the GD+ culture than in the GD− culture. $^{****}P < 0.01$, $^{***}P < 0.01$ and $^{**}P < 0.01$ significantly different from GD−; $^{\#\#\#\#}P < 0.01$ significantly different from GD+. (Data were collected $n = 3$ in each experiment, from three independent experiments, and indicated as the mean ± s.e.m); **c** Appearance of colonies in the control (GD−), and in the presence of GD+, PD and LY (×100); **d** Western blot analysis of MAPK and phosphorylated MAPK (pMAPK). Gonocytes were cultured for 4 days in the presence of GDNF. As a control cells were starved for 16 h without GDNF and treated with no chemicals, PD and LY for 20 min; **e** Estimation of phosphorylated MAPK expression based on the western blot from three independent immunoblot experiments (mean ± s.e.m). The level of phosphorylated MAPK was significantly lower in PD-treated cells than in GD+-treated cells ($^{**}P < 0.01$). However, the level of phosphorylated MAPK was not significantly different in the absence of GD (GD−) and in the presence of LY

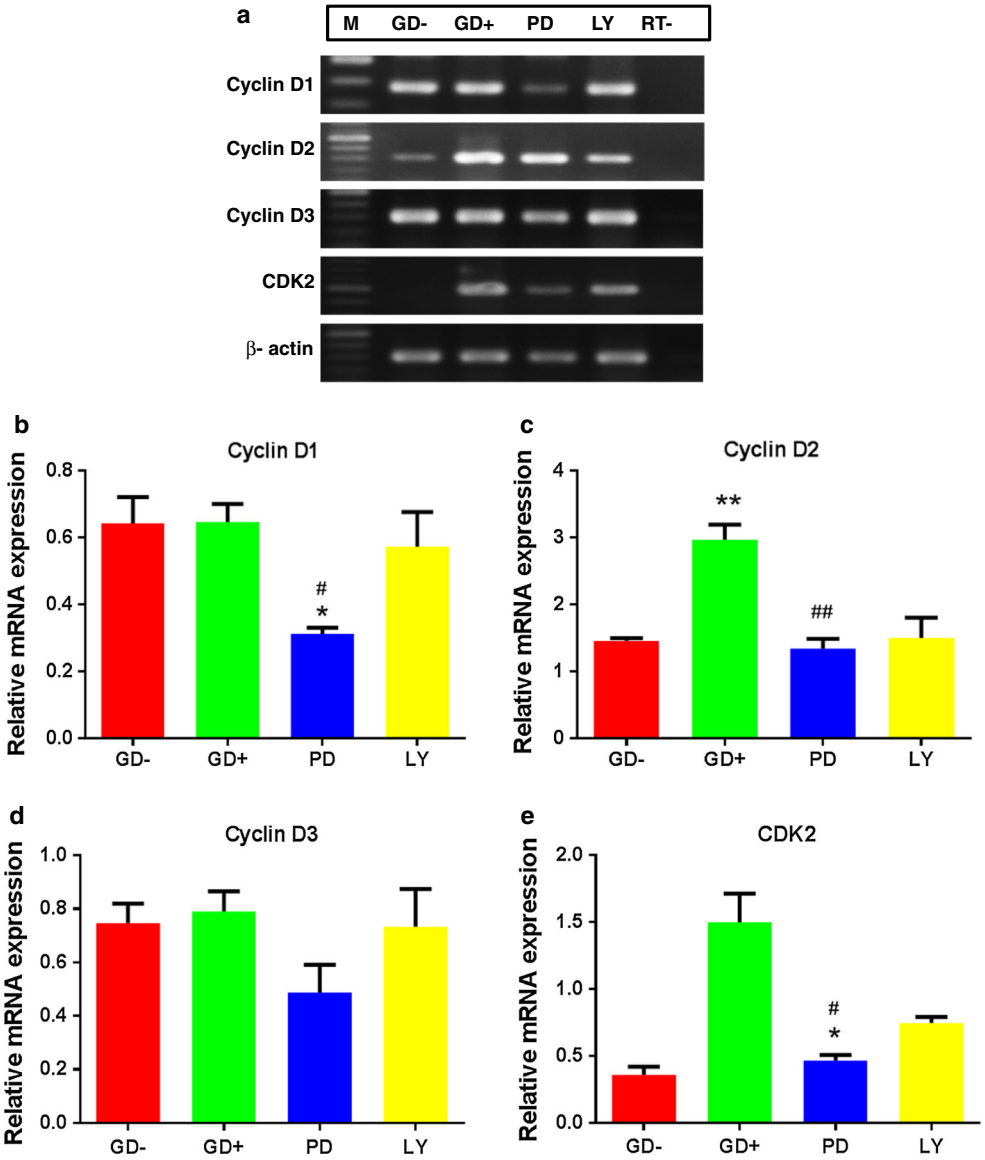

Fig. 3 Effect of inhibition of MAPK and PI3K signaling on the expression of cell cycle regulator genes. **a** RT-PCR analysis of cell cycle regulator genes (cyclin D1, cyclin D2, cyclin D3 and CDK2) and β-Actin as the housekeeping gene. Relative mRNA expression of cyclin D1 (**b**), cyclin D2 (**c**), cyclin D3 (**d**) and CDK2 (**e**) were examined in the presence of GDF or MAPK/PI3K inhibitors. Data represents 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+

RT–PCR (Fig. 3a). The addition of GDNF enhanced the expression of cyclin D2 and CDK2 (Fig. 3b, c, f). Expression of cyclin D1 and CDK2 was significantly reduced by the addition of PD to the culture medium (Fig. 3b, f). However, enhanced expression of cyclin D2 was significantly reduced by PD treatment (Fig. 3b). Expression of cyclin D3 was unaffected by the addition of GDNF or PD to the culture medium (Fig. 3d, e). Treatment with the LY inhibitor (PI3K signaling) did not influence expression of these genes.

Discussion

Signaling pathways that regulate self-renewal and differentiation of cultured germ cells have been well documented in mice [22]. However, the mechanisms of proliferation of cultured germ cells have not yet been elucidated in other species. Although several attempts have been made to develop a long-term culture system for bovine gonocytes, colony formation could not be obtained after subsequent passages [23, 24]. We previously established a long-term

culture system of bovine gonocytes for more than 1.5 months [25]. In this study culture conditions were stable enough to maintain cell survival and proliferation of bovine gonocytes. Mouse embryonic stem (ES)-like colonies appeared in the culture and expressed pluripotent marker genes (*OCT3/4* and *NANOG*) [25].

GDNF was shown to regulate self-renewal and differentiation of mouse SSCs [26]. GDNF signals act though the multi-component receptor complex comprised of GFRα-1 and RET tyrosin kinases in various cell types [27]. GFRα-1 and RET have also been recognized as spermatogonial markers expressed in gonocytes, SSCs and differentiated spermatogonia [28]. These co-receptors of GDNF-mediated signaling were shown to be necessary for self-renewal of germ cells in rodents [29]. GDNF-enhanced cell proliferation and colony formation of bovine gonocytes were reported by Aponte et al. [30, 31], which indicated that GDNF-mediated signaling was conserved in rodent and cattle germ cell cultures.

In the present study we showed that inhibition of MAPK pathways by the inhibitor PD98095 impaired cell proliferation and colony formation (Fig. 2a, b). The presence of GDNF significantly increased tyrosine phosphorylation of MAPK44/42 (Fig. 3d, e). This stimulation was blocked by treatment with PD98059 (Fig. 3d, e). These results indicate that activation of MAPK pathways is essential for self-renewal of cultured bovine gonocytes. In accordance with these results GDNF signals were previously shown to activate RET phosphorylation and subsequently activate MAPK pathways, which are essential for cell growth and proliferation of SSCs in mice [17]. Previous studies also demonstrated that FGF2, not GDNF, mediates activation of the MAPK pathway by upregulating the downstream targets ETV5 and Bcl6b in mouse germ cell cultures [32]. However, addition of FGF2 to our culture system enhanced somatic cell proliferation and induced differentiation of the gonocytes (unpublished data).

PI3K/AKT is known to play an important role in self-renewal of germ cells in mice via GDNF or FGF2 stimulation [33]. Activation of PI3K/AKT signaling in mouse germ cells was shown to be completely inhibited by the inhibitor LY294002, which impaired the self-renewal of cultured germ cells [15, 16]. However, activation of AKT alone was not sufficient for self-renewal of SSCs [15]. Src kinase is an alternative activator of PI3K pathways, which results in the upregulation of *N-myc* expression and promotes proliferation and self-renewal of mouse germ cells [14, 16]. Our results showed inhibition of PI3K/AKT signaling by LY294002 did not affect cell proliferation or colony formation of bovine germ cells. This indicated that AKT- or Src-mediated PI3K signaling did not play a significant role in self-renewal of cultured bovine gonocytes. This finding is in contrast to that reported in mice in which PI3K was shown to be the dominant signaling pathway.

Inhibition of MAPK and PI3K signaling was previously shown to result in downregulation of the pluripotency genes *OCT3/4*, *NANOG* and *SOX2* in human ES cell lines [34, 35], which indicated that these signaling pathways play essential roles in maintaining self-renewal and pluripotency of human ES cells. PI3K/AKT signaling was also shown to regulate expression of the self-renewal cascade genes *Bcl6b, Etv5, and Lhx1* in mice germ cell cultures [16]. Interestingly, the expression of *Oct3/4* was essential for survival of mouse germ cells, but was not influenced by GDNF and did not play a significant role in self-renewal [36]. However, expression of *OCT3/4* and *NANOG* was detected in cultured bovine gonocytes [25] and gonocytes in the testes of pigs [37] and cattle [25], suggesting that these pluripotent genes have roles in the maintenance and self-renewal of gonocytes in domestic species. In contrast, *Nanog* expression has not been detected in cultured bovine gonocytes or in the testes of mice [38]. Our previous report [25] demonstrated that strong expression of the pluripotency markers *OCT3/4* and *NANOG* in cultured bovine germ cells was associated with the appearance of mouse ES-like colonies. These results indicate that different expressions of transcription factors in mice and domestic species may lead to different regulatory mechanisms for self-renewal and colony formation of cultured germ cells. However, the role of these genes has to be elucidated further to understand the MAPK-mediated self-renewal of bovine gonocytes germ cells.

Activation of the extrinsic MAPK [17, 18] and PI3K [15] signaling pathways in mouse germ cells was previously shown to be involved in regulation of cell-cycle-related cyclin gene expressions. To understand the relationship between signaling pathways and self-renewal of cultured bovine gonocytes we analyzed the downstream genes potentially involved in cell cycle regulation. Cyclin D1 is essential for proceeding to the G1/S-phase of the cell cycle in the presence or absence of GDNF and is regulated by the MAPK pathway [39]. Expression of cyclin D1 has also been observed in proliferating germ cells and SSCs in mouse testes [40]. In this study the expression of cyclin D1 was significantly downregulated after the inhibition of MAPK signaling by PD (Fig. 3a, b), but was unaffected by the presence of GDNF. In contrast, cyclin D2 expression was significantly upregulated upon GDNF stimulation and inhibited upon pre-treatment with the MAPK inhibitor (Fig. 3a, c), which indicated that the MAPK pathway was involved in regulating the cell cycle of bovine gonocytes. Overexpression of cyclin D2 was previously shown to regulate self-renewal of germ cells and was mediated by Ras activation in mice [18]. CDK2 has been shown to be involved in controlling entry to the S-phase in association with cyclin A. CDK2 was upregulated in the presence of GDNF and controlled entry to the G1/S-phase of mouse

C18-4 germ cell lines via MAPK-mediated signaling [17]. In this study CDK2 expression was also significantly upregulated upon GDNF stimulation and the inhibition of MAPK signaling resulted in downregulation of CDK2 expression (Fig. 3a, e). Enhanced CDK kinase activity was previously shown to be essential for Ras-induced proliferation of cultured mouse germ cells [18]. Our results suggested that cell cycle-related genes were not influenced by inhibition of PI3K signaling. This is consistent with a previous report [16] in which the inhibition of PI3K signaling does not significantly affect changes in cyclin gene expression in cultured mouse germ cells.

Taken together, these findings reveal the unique and crucial role of MAPK signaling in maintaining self-renewal and colony formation of cultured bovine gonocytes. In contrast to our findings, cultured mouse germ cells require crosstalk between MAPK and PI3K signaling pathways for self-renewal. The downstream targets of MAPK signaling that ultimately influence the self-renewal of bovine gonocytes need to be determined in future experiments. The present study has revealed marked differences in the control of self-renewal and survival of cultured mice and cattle germ cells. These results will be useful for identifying optimal culture conditions for establishing a long-term culture system and germ-cell lines in domestic species.

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Conflict of interest Mahesh Sahare, Ayagi Otomo, Kana Komatsu, Naojiro Minami, Masayasu Yamada, and Hiroshi Imai declare that they have no conflict of interest.

Animal studies All institutional and national guidelines for the care and use of animals were followed.

Human rights This article does not contain any studies with human subjects performed by any of the authors.

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