Amino Acids (2011) 40:933–941
DOI 10.1007/s00726-010-0717-x

ORIGINAL ARTICLE

The promoting effect of retinoic acid on proliferation of chicken primordial germ cells by increased expression of cadherin and catenins

Minli Yu · Kun Guan · Caiqiao Zhang

Received: 11 May 2010 / Accepted: 3 August 2010 / Published online: 18 August 2010

Abstract Proliferation and cellular aggregation are both crucial features for survival and self-renewal of primordial germ cells (PGCs). Adhesive proteins play pivotal roles in cell–cell adhesion and signal exchanges under the influence of cytokines, growth factors and bioactive metabolites such as retinoic acid (RA). In this study, proliferation-promoting effect of RA on chicken PGCs was investigated by revealing changes in adhesive proteins E-cadherin and α/β catenins. PGCs were isolated from the genital ridge of 4-day-old chicken embryos and cultured on embryonic fibroblast feeder. RA (10^{-7}–10^{-5} M) increased PGCs aggregation and mRNA expression of E-cadherin and α/β-catenins. Furthermore, E-cadherin and β-catenin protein expression levels were increased by RA treatment. However, RA-elicited effect was significantly attenuated by a PKC inhibitor H_7. In addition, the number and area of PGC colonies were increased by RA treatment at 10^{-7}–10^{-5} M. Again, this increase was reduced by combined treatment of H_7. The proliferating effect of RA on PGCs was further confirmed by increased mRNA expression of cyclins, *CCND1* and *CCNE1*, and cyclin-dependent kinases 6 and 2, which are critical for G1–S progression in cell cycle. Moreover, flow cytometry analysis confirmed that RA-treated PGC populations displayed a significant increase in the proportion of S and G2 phase cells. Likewise, this stimulating action was hindered by combined H_7 treatment. These results indicate that RA, as a bioactive metabolite of vitamin A, may promote PGC proliferation and increase intercellular aggregation of PGCs via E-cadherin and α/β-catenins expression through the PKC signaling pathway.

Keywords Primordial germ cells · Retinoic acid · Cadherin · Catenin · Proliferation · Chicken

Introduction

Primordial germ cells (PGCs), as precursor cells developing into ova and spermatozoa in the embryonic stage, are model cells for genome research and transgenic application in a variety of vertebrates. In recent years, the limitation in the number and stability of cultured embryonic PGCs has attenuated the research on avian PGCs. Coordinating the proliferation and aggregation of cultured PGCs is a crucial step to obtaining sufficient surviving cells for diverse studies.

Intercellular connection plays an important role in both male and female germ and somatic cells. In the mouse, PGCs are connected to each other in the process of migrating to genital ridge until forming cell colonies (Nakamura et al. 1988). Cultured PGCs and oocytes separated from gonads form colonies (Pain et al. 1996). A variety of cell adhesion molecules (CAMs) play a direct role in the process of cell adhesion (Karagenc and Petitte 2000). Cadherin, as a member of the CAM family, makes great contribution to calcium-dependent cell adhesion (Nandadasa et al. 2009). Cadherin expression is closely associated with cell migration, cell selection, tissue formation and organ regeneration (Cavey et al. 2008). It was shown that E-cadherin, which was expressed on the surface of mouse

M. Yu and K. Guan contributed equally to this work.

M. Yu · K. Guan · C. Zhang (✉)
Key Laboratory of Animal Epidemic Etiology and Immunological Prevention of the Ministry of Agriculture, Department of Veterinary Medicine, College of Animal Sciences, Zhejiang University, No. 268 Kaixuan Road, Hangzhou 310029, People's Republic of China
e-mail: cqzhang@zju.edu.cn

PGCs, mediated cell-to-cell adhesion, and played an important role in PGC settlement within the genital ridge (Bendel-Stenzel et al. 2000). There are several types of cadherins with tissue-specific patterns. N-cadherin is expressed between germ and somatic cells, while P-cadherin is expressed between somatic cells. E-cadherin affects cell migration, proliferation and differentiation through intercellular and intracellular signal transduction pathways (Gomperts et al. 1994). In mice, E-cadherin appears from the eight-cell period and makes the cell junction tight, while its antibody inhibits this effect (Garcia-Castro et al. 1997). As the embryo develops, some mesodermal cells start expressing other kinds of cadherins instead of E-cadherin (De Felici et al. 2005). The α/β-catenins, which function as components of the cadherin complex, controls cell–cell adhesion and influence cell migration.

Cell adhesion is regulated by expression and interaction of the adhesive proteins under the influence of various local and circulating cytokines, growth factors and bioactive metabolites (Hulpiau and van Roy 2009). RA is a metabolite of vitamin A with essential biological activity. Chronic vitamin A deficiency induces a substantial delay in the rates of weight and height gain in both humans and experimental animals. However, in the vitamin A-deficient rat, all-*trans* RA partially caused reverting of the endogenous proteolysis and nitrogen metabolism (Esteban-Pretel et al. 2010). For many types of cells, RA plays an important role in regulating cell proliferation and differentiation (Vilhais-Neto and Pourquié 2008). For instance, RA is involved in the induction of neural differentiation, motor axon outgrowth and neural patterning (Maden 2007). RA also regulates the development of bone marrow-derived mesenchymal stem cells, ovarian cancer cells, liver cancer cells, HL-60 cells, etc. (Berggren et al. 1999). In human retinal pigment epithelial cells, RA remodels extracellular matrix and suppresses laminin-enhanced contractility of the cultured cells (Chang et al. 2009). RA treatment significantly induces chicken embryoid bodies to differentiate into dopamine and cholinergic neurons, astrocytes and oligodendrocytes (Wu et al. 2010). However, the effect of RA on avian PGCs adhesion and its mechanism has not yet been clarified.

RA is an essential component for cell–cell signaling during early vertebrate organogenesis and may act primarily in a paracrine manner and provide insight into the cell–cell signaling networks that control differentiation of pluripotent cells (Duester 2008). RA was detected in the lateral mesoderm, kidney and other parts of embryos, suggesting the potential roles of RA in chicken embryonic development (Helms et al. 1996). On the other hand, RA regulated Fgf8 expression in the tail spinal cord nerves to ensure normal differentiation, thus guaranteeing the normal development of the embryo's backbone (Koshimuzu et al. 1995). In the rat, chicken and zebrafish models, absence of RA signaling pathway led to left–right asymmetry of somite development, while RA and leukemia inhibitory factor (LIF) maintained mouse PGC survival and proliferation via promoting cell mitotic activity (Koubova et al. 2006). All-*trans* RA could regulate cell growth and differentiation (Bowles et al. 2006), and affect the transcription and translation process in various animals (Zorn and Sauro 1995). In mouse spleen cells, RA induced cytoplasmic protein kinase C (PKC) migration to the cell membrane, while PKC inhibitor H_7, sphingosine alcohol and star-shaped spores Su-13 were able to inhibit PKC phosphorylation that was induced by RA analogs 13-cis-RA and all-*trans* RA (Imam et al. 2001). Moreover, RA promoted the expression of PKCα through direct stimulation of transcription factors. Both RA and forskolin can regulate cell cycle through mediation of cyclin C (Makkonen et al. 2009). Conventional PKC protein contributes to this process via promoting activity of DNA binding with nuclear factor κB (Jung et al. 2005).

Amplication of PGCs is imperative for genetic manipulation and germline chimera production. Our previous studies showed that epidermal growth factor (EGF) and PKC activator could promote the proliferation of chicken PGCs (Ge et al. 2009; Tang and Zhang 2007). In this study, chicken PGC culture system was adopted to evaluate the effect of RA on germ cell aggregation by demonstration of the changes in expression of E-cadherin and α/β catenins mRNAs. In addition, we examined E-cadherin expression and β-catenin protein expression elicited by RA to investigate whether they were involved with RA action. At the same time, the change in cell proliferation was also detected by the colony number and area of PGC aggregates. For cell cycle analysis, flow cytometry was used to examine which cell cycle phase was affected by RA treatment. The results of these studies will facilitate elucidating the regulation of PGC proliferation by increased cell–cell adhesion through nutritional regulation such as vitamin A derivative RA.

Materials and methods

Isolation and culture of PGCs

Fertilized arbor acres broiler chicken eggs were incubated in egg incubator (Victoria SRL, Italy) at 38.5°C with 60% humidity for 4 days. The genital ridges were carefully dissected from the mesonephrose and dissociated in 0.05% trypsin/EDTA (E. Merck, Armstadt, Germany) solution at room temperature for about 5 min. After filtration through a 200-μm mesh, dissociated cells were washed three times with DMEM (Hyclone, Utah, USA) and then centrifuged at 1,000 rpm for 8 min. Cells were seeded at 1×10^6/well in

six-well culture plates (Costar, Corning Inc., USA) in DMEM supplemented with 5% fetal calf serum (FCS), 10 ng/ml LIF, 10 ng/ml basic fibroblast growth factor (Stemcell Inc, Canada), 0.1 mM MEM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. After 48 h of culture, PGC colonies were removed, dispersed and then subcultured on CEF in a medium supplemented with 5% FCS.

Treatment of the cultured cells with chemicals

Subcultured PGCs were treated with RA at 10^{-7}–10^{-5} M alone or in combinations with PKC inhibitor H_7 (10^{-7}–10^{-5} M), respectively. The chemicals were dissolved in ethanol and diluted with the medium. The final concentration of ethanol in the medium was ≤0.1%. The control received the vehicle only.

Determination of cell aggregation index

Cell aggregation index was designed to determine the cell adhesion activity, namely, intercellular adhesion molecule activity according to a method by Fujimoto et al. (1996). After 24 h of treatment, PGC colonies were removed, dispersed in HMF buffer containing 0.01% trypsin and 1 mM $CaCl_2$, and cultured on 1% albumin-coated 24-well plate. The plate was centrifuged at 90 rpm at 37°C for 60 min. Four different regions were randomly selected in each well and the number of single cells and cell colonies were counted with a digital video camera (Pixera Pro 150ES, USA). Cell aggregation index was defined as the ratio of the sum of the number of single cells and cell colonies to the number of all cells.

Immunocytochemical detection of SSEA-1 and SSEA-3

After fixation with 4% paraformaldehyde for 20 min, the subcultured cells were washed three times with PBS. The cells were incubated with blocking buffer containing 5% newborn bovine serum for 20 min, and then incubated with SSEA-1 (MC-480) and SSEA-3 (MC-631 from DSHB) as the first antibodies at 4°C overnight and at 37°C rewarming for 45 min. The primary antibody was replaced with normal serum in the negative control. TRITC or biotinylated anti-IgG (Santa Cruz, UC) was used as the second antibody. The nuclei were stained with DAPI. The fluorescence images were obtained with fluorescence microscopy (Olympus IX71, Tokyo, Japan).

RNA isolation and RT–PCR

Trizol was used for total RNA extraction and the reverse transcription kit (Fermentas) was used for cDNA synthesis. RT products were then used for PCR amplification under the following conditions: denaturing at 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. The last extension was 10 min at 72°C. Primers were designed according to GenBank accession gene, and Primer 5.0 software design primer sequence and PCR product length are listed in Table 1. Amplified products were verified by 1.5% agarose gel electrophoresis.

Western blot analysis

Western blotting was performed according to a conventional method. PGCs were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid and 0.1% SDS) supplemented with protease inhibitors (Complete; Roche) and 0.2 nM PMSF. For β-catenin immunoblotting, the nuclear protein of PGCs was purified by the methods of De Felici and Siracusa (1982). Proteins (30 μg) were then subjected to SDS–PAGE, transferred to a PVDF membrane (Millipore, USA) and blocked with TBST/5% nonfat milk. Western blots were probed with the respective primary antibodies: β-catenin antibody (1:4,000), E-cadherin (1:500) and β-actin antibody (1:5,000) (Novus Biologicals, USA). After incubation with

Table 1 Primers for PCR analysis

Genes	Primer sequences (5′–3′)	Accession no.	Product size (bp)
E-cadherin	GGT GAT GGA CCA GAA CGA GCG GCT CTT GGT CTC ATA	NM_001039258.1	598
α-catenin	GCA TTT CTT CGT CAC CCT GGT CTC CAG GAA CGA ATC	NM_205136.1	513
β-catenin	ATC AAG ATA CCC AGC GAC G GCC AGA GTG GAA AGA ACG	NM_205081.1	536
ACTB	ACG TCG CAC TGG ATT TCG AGn TGT CAG CAA TGC CAG GGT AC	NM_205518	282
CCND1	CTG CTC AAT GAC AGG GTG C TCG GGT CTG ATG GAG TTG T	NM_205381	341
CCNE1	ACC TAA AAT GAG AAC AAT CC GGC AAC AAT ACC TCG TAA A	NM_00103158	381
CDK2	ACT GCT GTG GAC ATC TGG A CTT GTT GGG ATC GTA GTG C	EF182713	276
CDK6	CCG ACC AAC AGT ATG AGT GCG GAA AAT CCA GTC CCC GAA ACA	NM_001007892	381

the secondary antibodies (anti-mouse or anti-rabbit IgG) conjugated with horseradish peroxidase (Santa Cruz, CA, USA) for 1 h at room temperature, immunostained bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Flow cytometry analysis

About 10^6 PGCs were pelletted by centrifugation at 400*g* for 5 min, and then washed twice in 1 ml iced PBS. The resuspended cells were transferred to 4 ml of absolute ethanol at −20°C by pipetting the cell suspension slowly into the ethanol while vortexing at top speed, leaving the cells in ethanol at −20°C for 30 min. After being pelletted and washed three times in PBS, fixed cells were treated with 100 μg/ml RNase A (Invitrogen) at 37°C for 20 min. Subsequently, cells were incubated in staining solution (10 μg/ml PI and 0.1% Trition X-100 in PBS) for 15 min in a dark room and analyzed by flow cytometry (BD FACS CELLULAR) in the presence of the dye.

Statistical analysis

For the data, SAS (9.0 version) software GLM process of analysis of variance and Duncan's multiple analysis and comparison were used. $P < 0.05$ was considered to be significantly different.

Results

Characterization of PGCs

After culture, the PGCs began proliferation and colonized together to become cell aggregates. Finally, the PGCs formed compact colonies (Fig. 1a–i). PGCs were identified by positive staining for SSEA-1 immunofluorescence (Fig. 1a, d, g) and the nuclei were stained with DAPI (Fig. 1b, e, h). Cultured PGC colonies were also identified by SSEA-1/3 immunocytochemical staining (Fig. 1j, k). Slices of gonadal tissue from 5.5-day-old chicken embryo were stained by SSEA-1 immunofluorescence method, with significant cell-surface red fluorescence for chicken PGC clusters (Fig. 1l).

Effect of RA on PGC adhesion

Aggregation index and expression of E-cadherin, α-catenin and β-catenin of PGCs were determined to detect the impact of RA on PGC aggregation. RA (10^{-7} M) reduced the aggregation index from 0.67 to 0.60 with about 10% decrease, while in 10^{-6} M RA-treated cells, the aggregation index was reduced to 0.37 with about 45% decrease (Fig. 2a). No further decrease in the aggregation index was achieved after higher RA treatment (10^{-5} M). At the same time, the mRNA expression of adhesion proteins was altered. Compared with the control, after treatment with RA at 10^{-7}, 10^{-6} and 10^{-5} M, the increase of E-cadherin mRNA expression was 5% ($P > 0.05$), 18 and 21% ($P < 0.05$), respectively. While α-catenin mRNA was increased by 8, 22 and 24%, β-catenin was increased by 7, 25 and 28.5%, respectively ($P < 0.05$, Fig. 2b, c).

Effect of H_7 on RA-induced PGCs adhesion

Though the PKC inhibitor H_7 imposed no significant effect on RA-stimulated reduction of aggregation index of PGCs at 10^{-7} M, higher H_7 at 10^{-6} and 10^{-5} M significantly attenuated the RA-elicited PGC aggregation (Fig. 3a). Simultaneous treatment of H_7 at 10^{-6} and 10^{-5} M remarkably attenuated the effect of RA on mRNA expression of adhesive proteins E-cadherin, α-catenin and β-catenin (Fig. 3b, c).

Involvement of E-cadherin and β-catenin in RA-induced cell proliferation

E-cadherin and β-catenin expression levels were increased by RA addition (Fig. 4), but RA-induced intracellular translocation of β-catenin was inhibited by H_7 pre-treatment (Fig. 4). E-cadherin expression level was also decreased by H_7 addition. Immunoblotting analysis indicated that the ability of primordial germ cells to form aggregates was increased in the presence of RA, compared with the control. However, simultaneous treatment of H_7 caused a significant decrease in the PGC colony number and markedly reduced their ability to form colonies in vitro.

Effect of RA and H_7 on PGC proliferation

After treatment with RA at 10^{-7}–10^{-5} M for 24 h, the number and area of PGC colonies were increased in a dose-dependent manner (Fig. 5). However, the RA-elicited PGC proliferation was reduced by the combined treatment of H_7 and the three-dimensional morphology of PGC colonies was weakened by H_7 (Fig. 6).

To further confirm the effect of RA on PGC proliferation, we examined the effect of RA on mRNA expression of cyclins, *CCND1* and *CCNE1*, cyclin-dependent kinase 6 (*CDK6*) and *CDK2*, which are considered to be critical factors in G1-S progression in cell cycle. Treatment with RA at 10^{-6} M increased the mRNA expression of all four genes significantly ($P < 0.05$, Fig. 7). This effect was hindered by combined H_7 treatment.

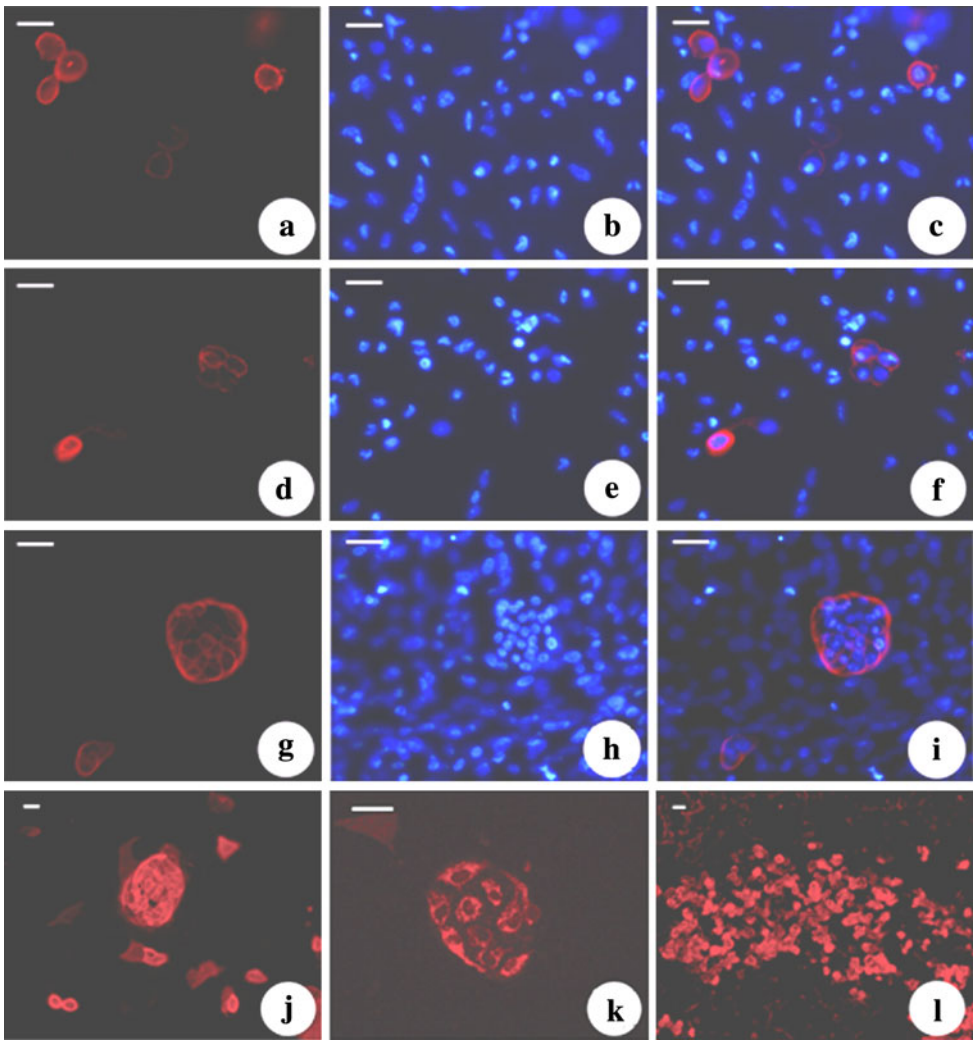

Fig. 1 Identification of PGCs. Immunocytochemical staining of SSEA-1 (**a**, **d**, **g**); nuclei were counterstained with DAPI (**b**, **e**, **h**) and then merged (**c**, **f**, **i**). Immunocytochemical staining of SSEA-1 (**j**) and SSEA-3 (**k**). Immunohistochemical staining of SSEA-1 in 5.5-day gonadal tissue slices (**l**). *Scale bar*, 20 μm

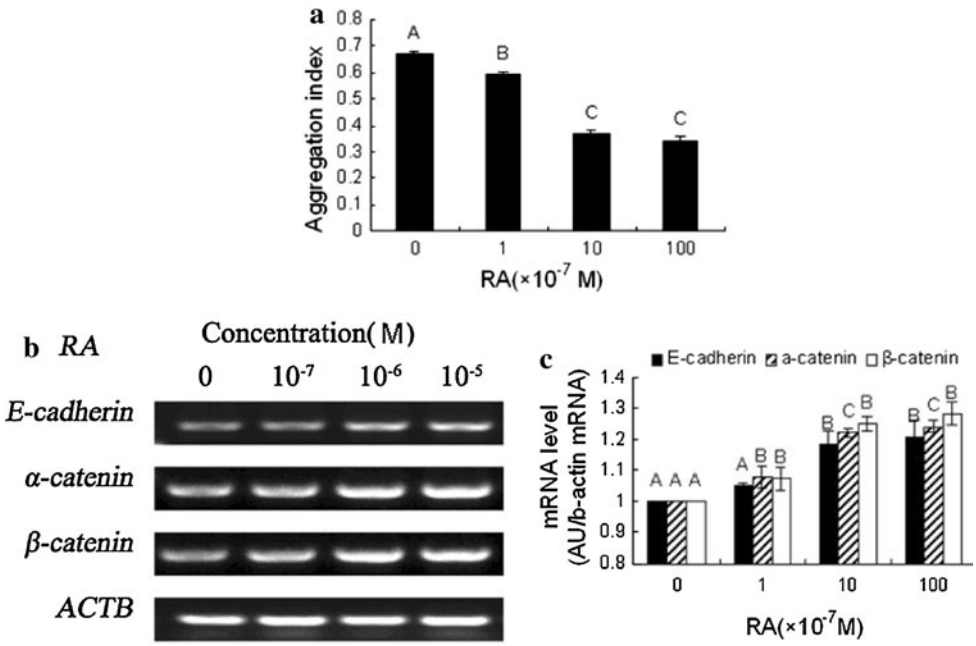

Fig. 2 Effect of RA on PGCs aggregation index and mRNA expression of adhesive proteins. **a** Change in PGCs aggregation index after RA treatment for 24 h. **b**, **c** Effect of RA on mRNA expression of E-cadherin and α- and β-catenin in PGCs. Values are the mean ± SEM ($n = 4$) determined from densitometry (*left*) relative to *ACTB*. *Bars* with different superscripts are statistically different

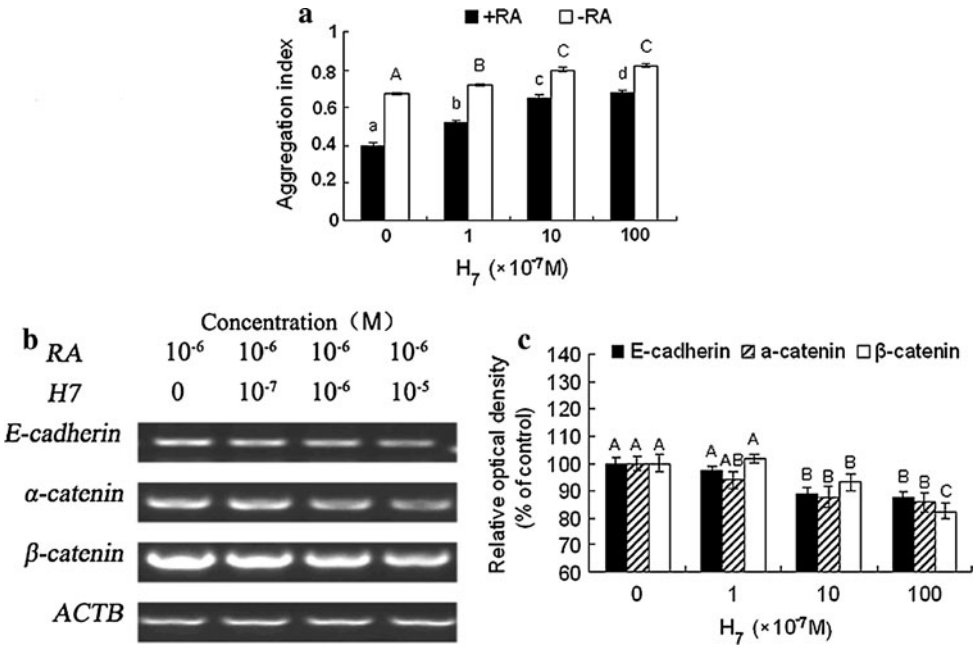

Fig. 3 Effect of H_7 on RA-stimulated cell aggregation index of PGCs. **a** Change in PGCs aggregation index after RA and H_7 treatment for 24 h. **b**, **c** Effect of H_7 on RA-stimulated mRNA expression of E-cadherin and α- and β-catenin in PGCs. Values are the mean ± SEM ($n = 4$) determined from densitometry (*left*) relative to *ACTB*. *Bars* with different superscripts are statistically different

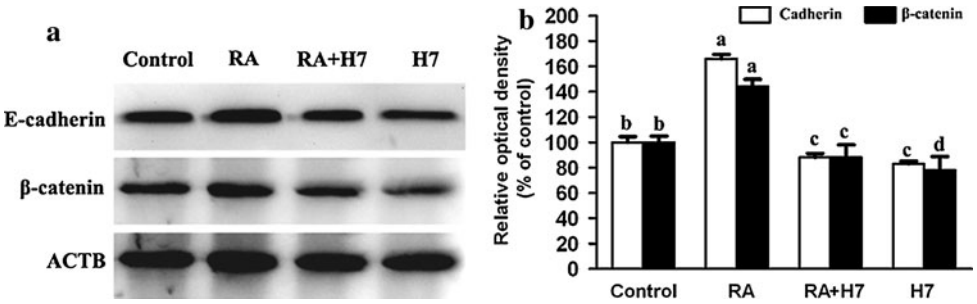

Fig. 4 Involvement of E-cadherin and β-catenin in RA-induced PGC proliferation. PGC were treated with H_7 or without 10^{-6} M RA for 24 h, then E-cadherin and β-catenin protein expression levels were detected by Western blot. Each example shown is representative of three independent experiments

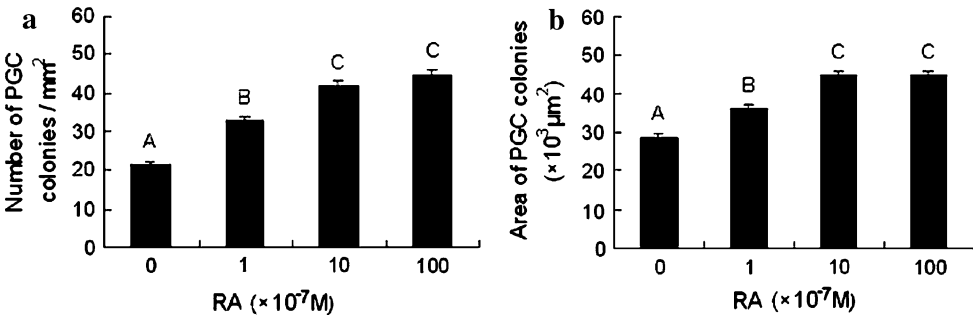

Fig. 5 Effect of RA on proliferation of PGCs assessed by changes in number (**a**) and area (**b**) of PGC colonies after incubation with RA (10^{-7}–10^{-5} M) for 24 h. Values are the mean ± SEM ($n = 4$). *Bars* with different superscripts are statistically different

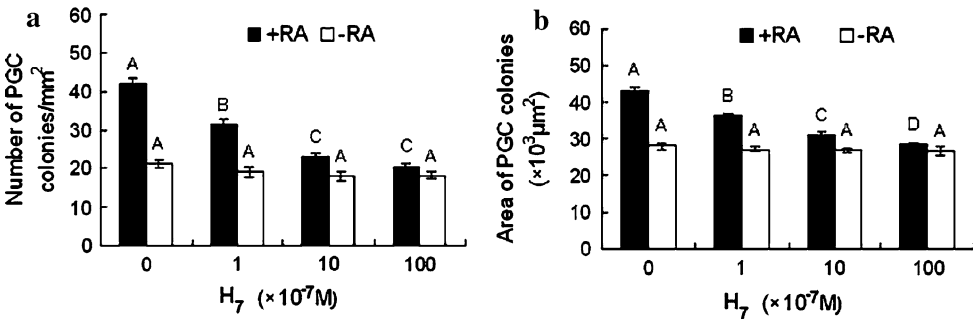

Fig. 6 Effect of H_7 on RA-induced proliferation of PGCs assessed by changes in the number (**a**) and area (**b**) of PGCs colonies after 24 h of treatment. Values are the mean ± SEM ($n = 4$). *Bars* with different superscripts are statistically different

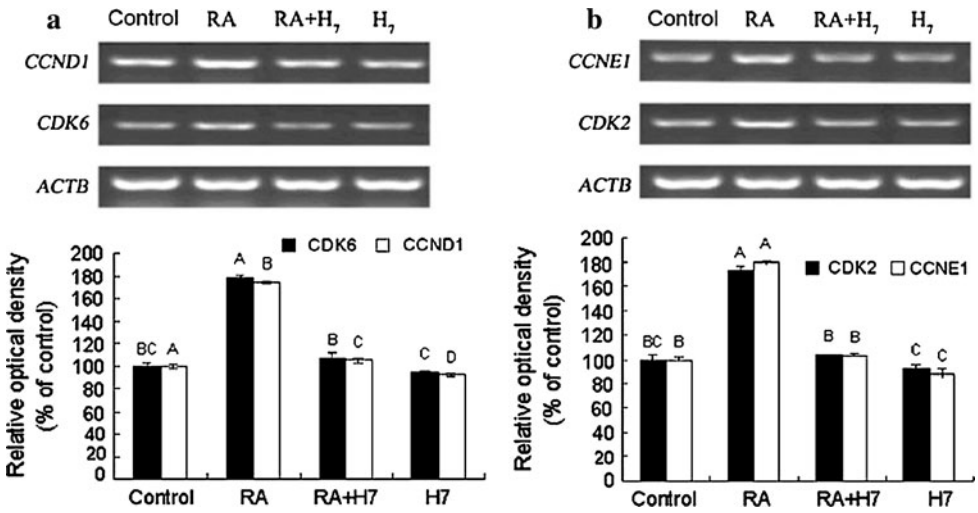

Fig. 7 Expression of cell cycle regulatory genes in cultured PGCs after 24 h of treatment with RA (10^{-6} M) and H_7 (10^{-6} M) alone or in combination. **a** Expression of *CCND1* and *CDK6*. **b** Expression of *CCNE1* and *CDK2*. **c**, **d** Mean ± SEM of three experiments for each condition determined from densitometry (*top*) relative to *ACTB*. *Bars* with different superscripts are statistically different

Flow cytometric analysis of PGCs

The result of flow cytometric analysis exhibited a clear increase of cells in S and G2 phase (from 23.88% in the control to 33.83% in RA-treated cells); while the proportion reduced to 16.45% in the RA + H_7 and 14.84% in the H_7 group, respectively (Fig. 8). The increased number of cells in the S and G2 phases was always paralleled by a reduction of the proportion of cells in the G1 phase (76.12% down to 66.17%, respectively, Fig. 8a, b). As shown in Fig. 8, combined treatment of H_7 with RA significantly decreased the percentage of S and G2 phase cells and increased the percentage of G1 phase cells (Fig. 8c, d).

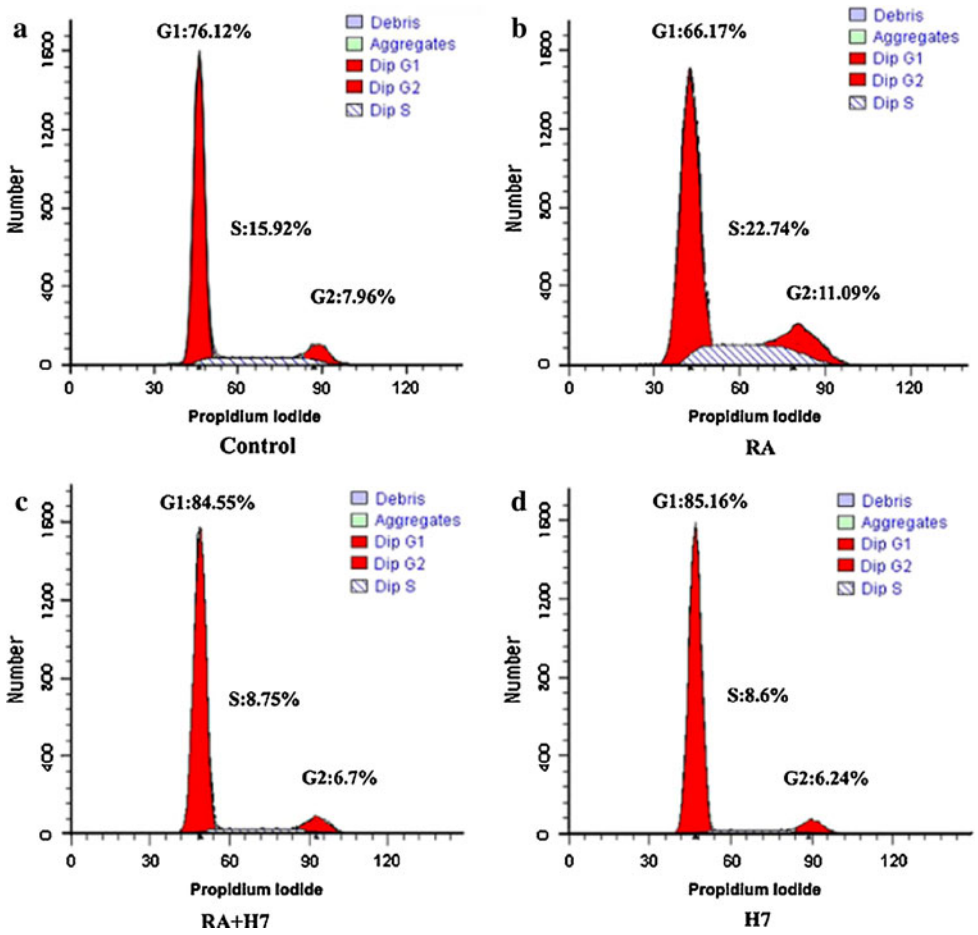

Fig. 8 Flow cytometric analysis of cell cycle in chicken PGCs after RA treatment. Cultured PGCs were treated with RA alone or with H_7 for 24 h. Then the percentage of each cell cycle stage was indicated for the proliferating PGC populations. To analyze cell cycle status, PI staining was used to quantify DNA content of individual cells. The cell cycle status of PGC populations analyzed using ModFit LT is shown. A minimum of 10,000 PGCs were analyzed for each sample. The experiment was repeated three times independently

Discussion

As a metabolite of vitamin A, RA plays an important role in the regulation of cell growth, differentiation, apoptosis and other bioactivities (Duester 2008). Vitamin A is involved in the generation of the seminiferous epithelium and the spermatogenic wave cycle (Hogarth and Griswold 2010). All-*trans* RA, as a physiological metabolite of RA compounds in vivo, combines with the RA receptor to create or regulate transcription genes, thereby regulating cell proliferation and differentiation. RA plays diverse roles in various target cells and organs, with the mechanisms of nuclear retinoid receptor binding and protein modifications (Takahashi 2010). In this study, the proliferating effect of RA on cultured chicken PGCs was revealed. After treatment of RA at 10^{-7}–10^{-5} M, both the number and area of PGC colonies were increased in a dose-dependent manner.

Besides the promoting action on PGCs, numerous physiological functions of RA have been reported. RA regulates the development of bone marrow-derived mesenchymal stem cells, ovarian cancer cells, liver cancer cells and HL-60 cells (Berggren et al. 1999). In the neuroblastoma, RA reduced the migration and invasiveness of the cell components of SK-N-SH cells (Messi et al. 2008). Regarding the diverse effects of RA, the mechanisms of the actions of RA have not yet been clarified. Many studies show that transglutaminases play an important role in extracellular matrix stabilization needed for cell differentiation. In the process of rat incisor cell regeneration, it was found that transglutaminases contributed to the connection between mesenchyme, epithelium and extracellular matrix as a complementary signal pathway (Caccamo et al. 2009). In liver cells, RA, as an inducer of transglutaminase, improved the formation of Gln-Lys bonds by increasing the catalysis effect of transglutaminase, thus regulating DNA synthesis and strengthening cellular connection and stability of the cell structure (Ohtake et al. 2008).

Nevertheless, only a few studies have examined its effect on germ or stem cells, and the mechanism of RA in regulating the development of stem cells remains largely unknown. In this study, the stimulating effect of RA on cellular adhesion and proliferation of PGCs was evaluated and PKC signaling pathway was activated in this action. A study of RA revealed that it acted against teratogenic cancer cells and psoriasis fibroblasts by regulating the activity of cAMP-dependent PKA. RA also works by activating PKC in melanoma cells and HL-60 cells. In rabbit and mouse spleen cells, all-*trans* RA decreased soluble PKC protein fragments and increased PKC cell membrane fragments through the PKC signaling pathway (Imam et al. 2001). Both RA and 14-hydroxy-4,14-anti-retinol activate the PKC signaling pathway by regulating the structural domains of Raf and PKC proteins (Hoyos et al. 2000). Our study confirmed that PKC activation was involved in RA-induced chicken PGC proliferation.

Cells within tissues adhere directly to one another through CAMs that mediate adhesive interactions between cells through their extracellular domains. As a widely studied CAM, cadherin represents a key molecule in cell–cell adhesion and cell signaling, and plays a critical role during tissue differentiation. The exoplasmic domains of E-cadherin dimers clustered on adjacent cells form Ca^{2+}-dependent homophilic interactions, and the cytosolic domains bind directly or indirectly to multiple adapter proteins such as catenins that connect the junctions to actin filaments of the cytoskeleton and participate in intracellular signaling pathways (Gumbiner 1996). E-cadherin that is expressed on chicken PGCs plays an essential role in migration and settlement and enhances PGC-to-PGC adhesion. In the process of PGC migration to the genital ridge and settlement, PGCs adhere to each other or exist as colonies. In culture, PGCs gradually form cell aggregates from single cells to accelerate the proliferation. Therefore, it may be predicted that cell-to-cell adhesion between PGCs plays a pivotal role for cell survival and proliferation. To better evaluate adhesion between PGCs, the aggregation index was determined in PGCs after RA treatment. HMF buffer containing 0.01% trypsin and 1 mM $CaCl_2$ was used to disperse cell colonies (Fujimoto et al. 1996). In this experiment, RA strengthened adhesion between PGCs through increased mRNA expression of E-cadherin and α/β catenins in a dose-dependent manner and enhanced protein expression of E-cadherin and β-catenin.

Besides analysis of the adhesive protein expression, the change in cell cycle-regulating genes including cyclins and their catalytic partners, the CDKs, was also examined. The results manifested that RA treatment increased mRNA expression of cyclins, *CCND1* and *CCNE1*, and *CDKs 6* and *2*. These genes are critical for G1–S progression in cell cycle. For cell cycle analysis, flow cytometry revealed a dramatic increase of cells accumulated at S and G2 phases upon RA treatment. However, this stimulating action was attenuated after PKC inhibition by H_7 treatment.

In conclusion, the proliferating effect of RA was revealed in cultured chicken PGCs. This stimulating action involved the increased expression of the adhesive proteins E-cadherin and α/β catenins, and elevated proportion of cells at S and G2 phases. The RA-elicited effect was attenuated by PKC inhibition. Increased cell proliferation was accompanied by elevated mRNA expression of cyclins, *CCND1* and *CCNE1*, and *CDKs 6* and *2*. These results indicate that RA, as a bioactive metabolite of vitamin A, may promote PGC proliferation and strengthen intercellular aggregation of PGCs via E-cadherin and α/β-catenins

expression through the PKC signaling pathway in chicken PGCs.

Acknowledgments This study was supported by the National Natural Science Foundation of China (No. 30871843) and Zhejiang Bureau of Science and Technology (2008C22040). We thank Weidong Zeng and Chutian Ge for help in the experiment. We also thank Ms. Jinhui Li, the technician of 985-Institute of Agrobiology and Environmental Sciences of Zhejiang University, for the assistance in using the BD FACS CELLULAR.

References

Bendel-Stenzel MR, Gomperts M, Anderson R, Heasman J, Wylie C (2000) The role of cadherins during primordial germ cell migration and early gonad formation in the mouse. Mech Dev 91:143–152

Berggren K, McCaffery P, Dräger U, Forehand CJ (1999) Differential distribution of retinoic acid synthesis in the chicken embryo as determined by immunolocalization of the retinoic acid synthetic enzyme, RALDH-2. Dev Biol 210:288–304

Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S et al (2006) Retinoid signaling determines germ cell fate in mice. Science 312:596–600

Caccamo D, Di Mauro D, Condello S, Currò M, Cutroneo G, Anastasi GP et al (2009) Expression pattern of transglutaminases in the early differentiation stage of erupting rat incisor. Amino Acids 36:49–56

Cavey M, Rauzi M, Lenne PF, Lecuit T (2008) A two-tiered mechanism for stabilization and immobilization of E-cadherin. Nature 453:751–756

Chang YC, Kao YH, Hu DN, Tsai LY, Wu WC (2009) All-*trans* retinoic acid remodels extracellular matrix and suppresses laminin-enhanced contractility of cultured human retinal pigment epithelial cells. Exp Eye Res 88:900–909

De Felici M, Siracusa G (1982) Survival of isolated, fully grown mouse ovarian oocytes is strictly dependent on externa calcium. Dev Biol 92:539–543

De Felici M, Scaldaferri ML, Farini D (2005) Adhesion molecules for mouse primordial germ cells. Front Biosci 10:542–551

Duester G (2008) Retinoic acid synthesis and signaling during early organogenesis. Cell 134:921–931

Esteban-Pretel G, Marin MP, Cabezuelo F, Moreno V, Renau-Piqueras J, Timoneda J et al (2010) Vitamin A deficiency increases protein catabolism and induces urea cycle enzymes in rats. J Nutri 140:792–798

Fujimoto J, Ichigo S, Hori M, Morishita S, Tamaya T (1996) Progestins and danazol effect on cell-to-cell adhesion, and E-cadherin and alpha- and beta-catenin mRNA expressions. J Steroid Biochem Mol Biol 57:275–282

Garcia-Castro MI, Anderson R, Heasman J, Wylie C (1997) Interactions between germ cells and extracellular matrix glycoproteins during migration and gonad assembly in the mouse embryo. J Cell Biol 138:471–480

Ge CT, Yu ML, Petitte JN, Zhang CQ (2009) Epidermal growth factor-induced proliferation of chicken primordial germ cells: involvement of calcium/protein kinase C and NFKB1. Biol Reprod 80:528–536

Gomperts M, Garcia-Castro M, Wylie C, Heasman J (1994) Interactions between primordial germ cells play a role in their migration in mouse embryos. Development 120:135–141

Gumbiner BM (1996) Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 84:345–357

Helms JA, Kim CH, Eichele G, Thaller C (1996) Retinoic acid signaling is required during early chick limb development. Development 122:1385–1394

Hogarth CA, Griswold MD (2010) The key role of vitamin A in spermatogenesis. J Clin Invest 120:956–962

Hoyos B, Imam A, Chua R, Swenson C, Tong GX, Levi E et al (2000) The cysteine-rich regions of the regulatory domains of Raf and protein kinase C as retinoid receptors. J Exp Med 192:835–845

Hulpiau P, van Roy F (2009) Molecular evolution of the cadherin superfamily. Int J Biochem Cell Biol 41:349–369

Imam A, Hoyos B, Swenson C, Levi E, Chua R, Viriya E et al (2001) Retinoids as ligands and coactivators of protein kinase C alpha. FASEB J 15:28–30

Jung JG, Kim DK, Park TS, Lee SD, Lim JM, Han JY (2005) Development of novel markers for the characterization of chicken primordial germ cells. Stem Cells 23:689–698

Karagenc L, Petitte JN (2000) Soluble factors and the emergence of chick primordial germ cells in vitro. Poult Sci 79:80–85

Koshimuzu U, Watanabe M, Nakatsuji N (1995) Retinoic acid is a potent growth activator of mouse primordial germ cell in vitro. Dev Biol 168:683–685

Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD, Page DC (2006) Retinoic acid regulates sex-specific timing of meiotic initiation in mice. Proc Natl Acad Sci USA 103:2474–2479

Maden M (2007) Retinoic acid in the development, regeneration and maintenance of the nervous system. Nature Rev Neurosci 8:755–765

Makkonen KM, Malinen M, Ropponen A, Väisänen S, Carlberg C (2009) Cell cycle regulatory effects of retinoic acid and forskolin are mediated by the cyclin C gene. J Mol Biol 393:261–271

Messi E, Florian MC, Caccia C, Zanisi M, Maggi R (2008) Retinoic acid reduces human neuroblastoma cell migration and invasiveness: effects on DCX, LIS1, neurofilaments-68 and vimentin expression. BMC Cancer 8:30

Nakamura M, Kuwana T, Miyayama Y, Fujimoto T (1988) Extragonadal distribution of primordial germ cells in the early chick embryo. Anat Rec 222:90–94

Nandadasa SA, Tao QH, Heasman J, Wylie C (2009) Cadherin function: right place, right time. Dev Biol 331:442–455

Ohtake Y, Maruko A, Ohishi N, Kawaguchi M, Satoh T, Ohkubo Y (2008) Effect of retinoic acid on transglutaminase and ornithine decarboxylase activities during liver regeneration. Cell Biochem Funct 26:359–365

Pain B, Clark ME, Shen M, Nakazawa H, Sakurai M, Samarut J et al (1996) Long-term in vitro culture and characterization of avian embryonic stem cells with multiple morphogenetic potentialities. Development 122:2339–2348

Takahashi N (2010) Vitamin A in health. J Health Sci 56:144–153

Tang XY, Zhang CQ (2007) Activation of protein kinases A and C promoted proliferation of chicken primordial germ cells. Anim Reprod Sci 101:295–303

Vilhais-Neto GC, Pourquié O (2008) Retinoic acid. Curr Biol 18:550–552

Wu YQ, Ge CT, Zeng WD, Zhang CQ (2010) Induced multilineage differentiation of chicken embryonic germ cells via embryoid body formation. Stem Cells Dev 19:195–202

Zorn NE, Sauro MD (1995) Retinoic acid induces translocation of protein kinase C (PKC) and activation of nuclear PKC (nPKC) in rat splenocytes. Int J Immunopharmacol 17:303–311