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Formation and activation induction of primordial follicles using granulosa and cumulus cells conditioned media

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Abstract

Fertility preservation of prepubertal girls subjected to invasive cancer therapy necessitates defining protocols for activation of isolated primordial follicles. Granulosa (GCs) and cumulus cells (CCs) play pivotal role in oocyte development. Although GCs and CCs share some similarities, they differ in growth factors production. The current study was conducted to evaluate the effects of GCs, CCs and their conditioned media on mice primordial follicles activation. One-day-old mice ovaries were subjected to 6-day culture with base medium (BM), GC conditioned medium (GCCM), GC coculture (GCCC), CC conditioned medium (CCCM) or CC coculture (CCCC). Follicular growth and primordial to primary follicle transition was observed during 6-day culture, and follicular activation rate tended to be greater in GCCM than other groups ($0.05 < P < 0.10$). On Day 6, the expression of phosphatase and tensin homolog (PTEN) in GCCM group was lower than that in BM group ($P = 0.020$), the expression of phosphoinositide-3-kinase was higher in CCCC group than BM, GCCM and CCCM groups ($P < 0.05$), and the expression of connexin 37 was greater in the CCCM group as compared with BM, GCCC, and CCCC groups ($P < 0.01$). In conclusion, the current study showed that condition medium of GCs could enhance in vitro activation of primordial follicles, probably through down-regulation of PTEN.

KEYWORDS
condition medium, granulosa cells, Pi3k, Pten, primordial follicles activation

1 | INTRODUCTION

Chemotherapy and radiotherapy could detrimentally impact ovarian primordial follicles leading to premature ovarian failure in cancer patients (Molina, Barton, & Loprinzi, 2005; Pérez-Andújar, Newhauser, Taddei, Mahajan, & Howell, 2013). Strategies for fertility preservation, including oocyte and embryo cryopreservation, require time for ovary stimulation as well as oocyte or embryo recovery, thus postponing oncotherapy, which would not be an option in patients afflicted with cancer (Fathi et al., 2017; Jeruss & Woodruff, 2009; Xiao et al., 2015). On the contrary, there is a major concern for cancer reoccurrence following ovarian tissue cryopreservation and autotransplantation due to the risk of contamination with malignant cells (Amorim & Shikanov, 2016; Bockstaele, Tsepelidis, Dechene, Englert, & Demeestere, 2011; Xiao et al., 2015). In this regard, in vitro culture of follicles could surpass the aforementioned techniques since it neither delays cancer treatment nor risks the reintroduction of malignant cells in the patients (Jeruss & Woodruff, 2009; Motamed et al., 2017; Xiao et al., 2015).

Discovery of factors activating primordial follicles during in vitro culture of ovarian tissue or isolated follicles could help increase the

number of growing follicles (Adhikari et al., 2012; Nelson, Telfer, & Anderson, 2012). Although in vitro activation of primordial follicles in pubertal women has been successfully achieved previously (Telfer, McLaughlin, Ding, & Thong, 2008), in vitro activation of primordial follicles in prepubertal girls requires to be defined (Luyckx et al., 2013; Telfer & Zelinski, 2013). In this context, coculture of ovarian tissue with somatic cells including granulosa and cumulus cells (CCs) has been adopted to stimulate primordial follicles activation and improve their growth (Casillas et al., 2014; Heidari, Malekshah, Parivar, Khanbabaei, & Rafiei, 2011; Z. Li et al., 2011; Ramesh et al., 2010; Tao et al., 2008). In addition, the conditioned media of granulosa or CCs have been indicated to enhance follicular growth (Abdel-Ghani, Shimizu, Asano, & Suzuki, 2012; Ling et al., 2008; Malekshah, Moghaddam, & Daraka, 2006; Zheng et al., 2012). In comparison to coculture with somatic cells, conditioned media have the advantage of avoiding intimate contact with some somatic cells, which could culminate in physiological damage and/or reinitiation of dormancy in follicles (Terrovitis, Smith, & Marbán, 2010). Moreover, conditioned media could be stored to be used whenever it is required (Malekshah et al., 2006).

Granulosa cells (GCs) are the primary somatic cells supporting the growth and development of oocytes from primordial to antral stage (Huang & Wells, 2010). With the follicular antrum formation, GCs differentiate into two anatomically and functionally distinct cells including mural GCs composing the wall of antral follicle and CCs surrounding the oocyte (Chian, Lim, & Tan, 2004; Gilchrist, Lane, & Thompson, 2008). Although GCs and CCs share some similarities, they are different in response to gonadotropins (Khamsi & Roberge, 2001) and secretion of growth factors, giving them specialized role in terms of oocyte development (Fatehi & Ebrahimi, 2015; Huang & Wells, 2010; Sadr, Ebrahimi, Shahhoseini, Fatehi, & Favaedi, 2015).

Phosphatase and tensin homolog (PTEN) is the main negative regulator of phosphoinositide 3-kinase (PI3K), thereby contributing to perpetuation of dormancy in primordial follicles (Kim, 2012; Zhang et al., 2017). Upon activation of PI3K pathway, the latency phase of primordial follicles terminates and the squamous GCs surrounding the oocyte gradually convert to cuboidal GCs, which govern the further development of activated ovarian follicle (Kim, 2012; Zhang et al., 2017). In this regard, the expression of connexin 37 (CX37), which is gap junction protein, has been observed to increased following follicular activation and is believed to facilitate communicative association of between GCs and oocyte (Teilmann, 2005; Veitch, Gittens, Shao, Laird, & Kidder, 2004).

Therefore, the aim of this study was to evaluate the effect of granulosa and CCs and their condition media on activation of 1-day-old mice primordial follicles and the expression of PTEN and PI3K as the main regulators of ovarian follicle activation.

2 | MATERIALS AND METHODS

2.1 | Animals

NMRI mice (Royan Institute, Tehran, Iran) were kept under controlled conditions (temperature: 20–25°C; humidity: 40–60%) with 12-hr light cycle. All procedures of current study were done under approval of Royan Ethics Committee (No. EC/93/1086).

2.2 | Experimental groups and study design

One-day-old animals ($n = 21$) were killed by decapitation (seven repeats) and all ovaries were removed. For control group, a total of seven ovaries were immediately fixed for both morphological (three ovaries) and molecular (four ovaries) evaluations and the remaining ovaries were randomly divided into experimental groups (seven ovaries per each group) and in vitro cultured during 6 days using (a) base medium (BM; in vitro culture control), (b) GCs conditioned medium (GCCM), or (c) CC conditioned medium (CCCM), or (d) cocultured with granulosa cells (GCCC) or (e) cumulus cells (CCCC). Ninety-six-well plates containing 200 μl α-minimum essential medium (α-MEM; Gibco, Paisley, UK) were used for in vitro culture. Every other day, 75% of the medium of each well was changed.

2.3 | Preparation of conditioned medium from granulosa cells

To produce conditioned medium, GCs were harvested from ovaries of 21–30-day-old NMRI mice, which were killed before excision of ovaries. Following excision, the ovaries were placed in α-MEM supplemented with 10% fetal bovine serum (FBS; Gibco), 50 μg/ml penicillin (Sigma-Aldrich, St. Louis, MI) and 50 μg/ml streptomycin (Sigma-Aldrich) under mineral oil (Sigma-Aldrich) and preantral follicles (100–120-μm diameter) were mechanically dissected from the surrounding tissue. Isolated follicles were cultured for 10–14 days in four-well plates containing 500 μl of the same medium supplemented with 10 mIU/ml recombinant follicle stimulating hormone (Merck, Darmstadt, Germany). The medium was changed 72 hr after culture commencement, at which GCs adhered to the bottom of plate while the follicles were suspending in the medium (Figure 1). Accordingly, the detached follicles were discarded and the remaining adherent cells were cultured up to passage 4 to prepare GC line. Condition media were harvested from passages 3 and 4 and were freshly used for in vitro culture of one-day-old mice ovaries.

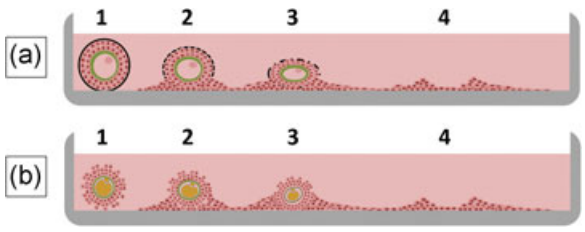

FIGURE 1 Granulosa and cumulus cells isolation and culture for providing conditioned medium. Granulosa cells were harvested from the ovaries of mice with 21–30 days old (a). Cumulus oocyte complexes (COCs) were collected from mice with 21–24 days old (b) [Color figure can be viewed at wileyonlinelibrary.com]

2.4 | Preparation of conditioned medium from CCs

Cumulus oocyte complexes (COCs) were collected from 21–24-day-old mice. To stimulate follicular development, mice received 7.5 IU/ml pregnant mare's gonadotropin (PMSG; Sigma-Aldrich) followed by 7.5 IU/ml human chorionic gonadotropin (hCG; Merck) 48 hr later. Eighteen hours after hCG injection, animals were killed and oviducts were collected and placed into α-MEM supplemented with 10% FBS, 50 μg/ml penicillin, and 50 μg/ml streptomycin under mineral oil. COCs were harvested by rupturing the ampulla using a sterile needle. COCs with ≥2 layers of intact CCs were further subjected to culture in four-well plates containing α-MEM supplemented with 10% FBS, 10 mIU/ml recombinant follicle stimulating hormone and 50 μg/ml penicillin, and 50 μg/ml streptomycin. As aforementioned for preparation of granulosa conditioned medium, the culture medium was changed 72 hr after the initiation of culture and concomitantly the detached follicles were eliminated (Figure 1). Then, the remaining cells were cultured up to four passages to prepare confluent CCs. Condition medium were harvested from passages 3 and 4 and were freshly used for in vitro organ culture of 1-day-old mice ovaries.

To prepare condition media, cells with 80–90% confluence were cultured with α-MEM supplemented with 10% FBS for 48 hr at 37°C. Afterwards, during passage 4, the medium was collected and centrifuged for 10 min at 4000*g*.

2.5 | Organ coculture of the mouse ovaries with GCs or CCs

GCs or CCs resulted from passage 3 were seeded at concentration of 20,000 cells into 96-well plates containing α-MEM supplemented with 10% FBS. When the cells reached the confluence of ≥70%, coculture of 1-day-old mice ovaries initiated.

2.6 | Anti-Müllerian hormone (AMH) immunofluorescence staining

Immunofluorescence staining of AMH was performed so as to substantiate that the cells used for coculture and preparation of conditioned media were putatively granulosa and CCs. GCs and CCs were seeded in four-well culture plate following washing with phosphate-buffered saline (PBS) to remove unattached cells. Initially, cells were fixed using 4% paraformaldehyde for 30 min, blocked with 5% goat serum in PBS for 1 hr at room temperature and coincubated with AMH antibody (1:100; GTX129593; GeneTex, Irvine, CA) overnight at 4°C. Then, cells were washed and incubated with anti-rabbit secondary antibody (1:500; ab96899; Abcam) for 1 hr at 37°C, followed by washing and staining with 4′,6-diamidino-2-phenylindole (DAPI) for 1 min. Preantral follicle served as the positive control for AMH immunofluorescence staining. The fluorescent signals were examined under a Nikon epifluorescence microscope (Eclipse 80i; Nikon, Tokyo, Japan). The number of AMH-positive cells were counted using ImageJ software (Wayne Rasband of the National Institutes of Health, Washington DC).

2.7 | Histological evaluation of the mouse ovaries

To calculate the rate of formation and activation of primordial follicles, fresh and in vitro cultured (three ovaries from each treatment) ovaries were fixed in bouin's and then in formalin. Then, they were dehydrated, embedded in paraffin, serially sectioned (6-μm-thick sections) and stained with hematoxylin and eosin. For each ovary, the sections were serially assessed using light microscope. Follicles were classified as primordial (one layer of flattened GCs around the oocyte), transitional (one layer of both flattened and cuboidal GCs), primary (a single layer of cuboidal GCs around the oocyte) or secondary (two or more layers of cuboidal GCs). Besides, follicular activation was defined as the proportion of all types of activated follicles including transitional, primary and secondary follicles.

2.8 | Comparison of messenger RNA (mRNA) gene expression by real-time polymerase chain reaction (PCR)

At the end of the culture period, the ovaries were collected and stored in RNA later at −80°C. Real-time PCR was done on RNA extracted from cultured whole ovaries to measure the levels of Cx37, Pten, and Pi3k (phosphoinositide-3-kinase regulatory subunit 5; Pik3r5) mRNA expression. Four ovaries from seven repeat culture wells from each group were subjected to RNA extraction. Total RNA was extracted using RNeasy Plus Micro Kit (Qiagen, Hilden, Germany), and was reverse-transcribed to complementary DNA (cDNA) using a standard oligo-dT reverse-transcription protocol in a reaction volume of 20 μl. From each cDNA sample, 2 μl was used as template for real-time PCR analysis. Each sample was run in duplicate. The Power SYBR Green PCR Master Mix (ABI) was used according to the manufacturer's instructions (Applied Biosystems, Warrington, UK). Real-time PCR was performed on an ABI step one plus thermocycler. The protocol was 40 cycles at 94°C for 10 min, 94°C for 30 s, and 60°C for 1 min. Fluorescent detection data were analyzed and normalized for Cx37, Pten, Pi3k mRNA levels to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. Data were expressed as Cx37, Pten, Pi3k, mRNA/GAPDH mRNA normalized to 1 day ovary.

2.9 | Western blot analysis for immunoblotting analysis

To evaluate the effect of experimental groups on PTEN and enzymatic activity of PI3K, western blot analysis was used to determine the protein level of PTEN and phosphorylated Akt (Akt-P), respectively. Whole ovaries ($n = 20$) were washed four times with cold PBS to remove any remaining culture media. Ovaries were then lysed by adding 50 μl 1× sample buffer (0.5 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue [BPB], 50 mM dithiothreitol [DTT], 1× cocktail protease inhibitor, and 1× cocktail phosphatase inhibitor). Supernatant total proteins were centrifuged at 14,000 RCF 4°C for 10 min, followed by transferring the supernatants to new tubes. Proteins extracted from ovaries were assayed by the BCA method (23227; Thermo Fisher Scientific, Waltham, MA). Total proteins (5 μg) were separated on 12%

polyacrylamide gels using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 100 V, 2 hr, followed by temperature controlled wet electrotransfer to Hybond polyvinylidene fluoride membranes at 15 V at 15°C, overnight. After a brief rinse with Mili-Q water (Millipore, Bedford, MA), membranes were blocked at room temperature for 2 hr with TBST containing 1% BSA and 0.1% Tween 20. The blots were incubated overnight with primary antibodies rabbit anti-PTEN (catalog no. 9559, at 1:1000; Cell Signaling Technology, Danvers, MA) and antibodies rabbit anti-Phospho-AKT (Thr308; Cell Signaling Technology, catalog no. 9275, at 1:1000) in TBST containing 0.2% BSA and 0.1% Tween 20. After the primary incubation, blots were washed three times in TBST containing 0.1% Tween 20. To detect the primary antibodies, blots were incubated 1 hr with HRP-conjugated donkey anti-rabbit secondary antibody (1:50,000) in TBST followed by three washes to exclude any nonspecific bands. Protein bands were detected by adding Super-Signal West Femto Maximum Sensitivity substrate (Rockford, IL). Chemiluminescent signals were captured and quantified by Alliance Q9 Advanced GelDoc system (Cleaver Scientific, Warwickshire, UK).

2.10 | Statistical analysis

Data were analyzed using general linear models procedure including repeated measures in the model. Binary variables including data associated with percentage of different categories of follicles as well as follicle activation rate were arcsine-transformed before analysis. All analyses were conducted in SAS version 9.4 (SAS Institute, Carry, NC). Differences at $p \leq 0.05$ and $0.05 < p < 0.10$ were considered statistically significant and tended to be statistically significant, respectively.

3 | RESULTS

3.1 | AMH level

Immunocytochemistry confirmed that granulosa and CCs used for culture and preparation of condition media in the current study were actively capable in AMH production. The results show a comparative higher activity in passage 4 cultured GCs against cultured CCs (Figure 2).

3.2 | Follicle formation, activation, and growth

Figure 3 shows histological sections of ovaries before and after in vitro culture. While formation of primordial follicles was found in all groups, their activation was observed only after in vitro culture. Despite the initial follicle growth to primary stage in some groups, in none of the treatments these follicles achieved the secondary stage (Figure 4).

After in vitro culture, the proportion of primordial follicles decreased compared to fresh control ovaries ($p < 0.0001$). On Day 6, the proportion of primordial follicles tended to be lower in the GCCM than CCCC group ($p = 0.083$; Figure 4). In all cultured groups, the proportion of transitional follicles increased on Day 6 compared with fresh control ($p < 0.01$). This proportion tended to be greater in GCCM than CCCC group ($p = 0.084$; Figure 4). In addition, the proportion of primary follicles did not change over the course of

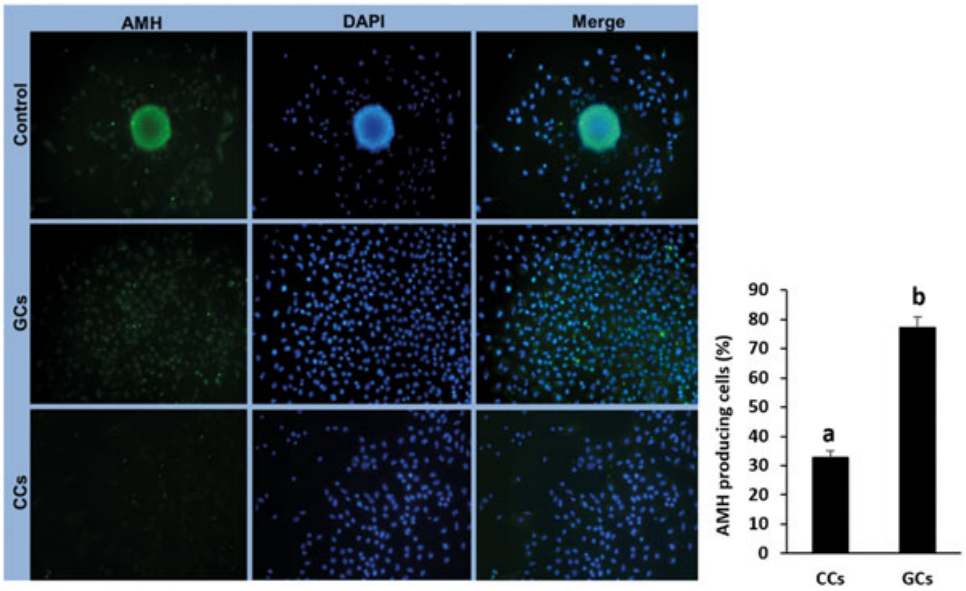

FIGURE 2 AMH immunofluorescence staining in passage 4 cultured GCs and CCs. Purified cells were examined with immunofluorescence method after four passages. The density of GCs and CCs was adjusted to approximately 70% confluence. Preantral follicle served as the positive control for AMH immunofluorescence staining. Green staining of AMH indicated the GCs and CCs origin. DAPI staining (blue) indicated the location of cell nuclei. The granulosa cells were stained positive for AMH and confirming the purity of GCs population and their activity which was clearer than CCs (a). Magnification: ×400 for all figures. Proportion of AMH-positive cells (b). AMH: anti-Müllerian hormone; CCs: cumulus cells; GCs: granulosa cells [Color figure can be viewed at wileyonlinelibrary.com]

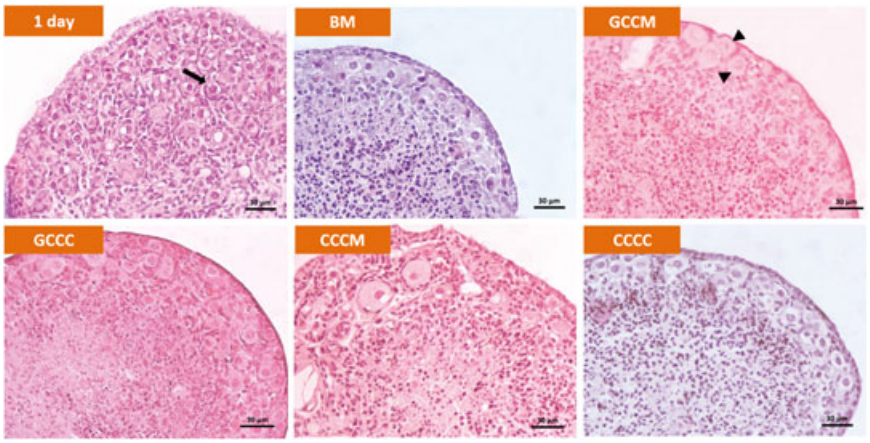

FIGURE 3 Fresh and cultured mouse ovaries was stained with hematoxylin and eosin. Noncultured fresh 1-day-old mouse ovary contains mainly forming primordial follicles (black arrow). BM: base medium; CCCC: cumulus cell coculture; CCCM: cumulus cell condition medium; GCCC: granulosa cell coculture [Color figure can be viewed at wileyonlinelibrary.com]

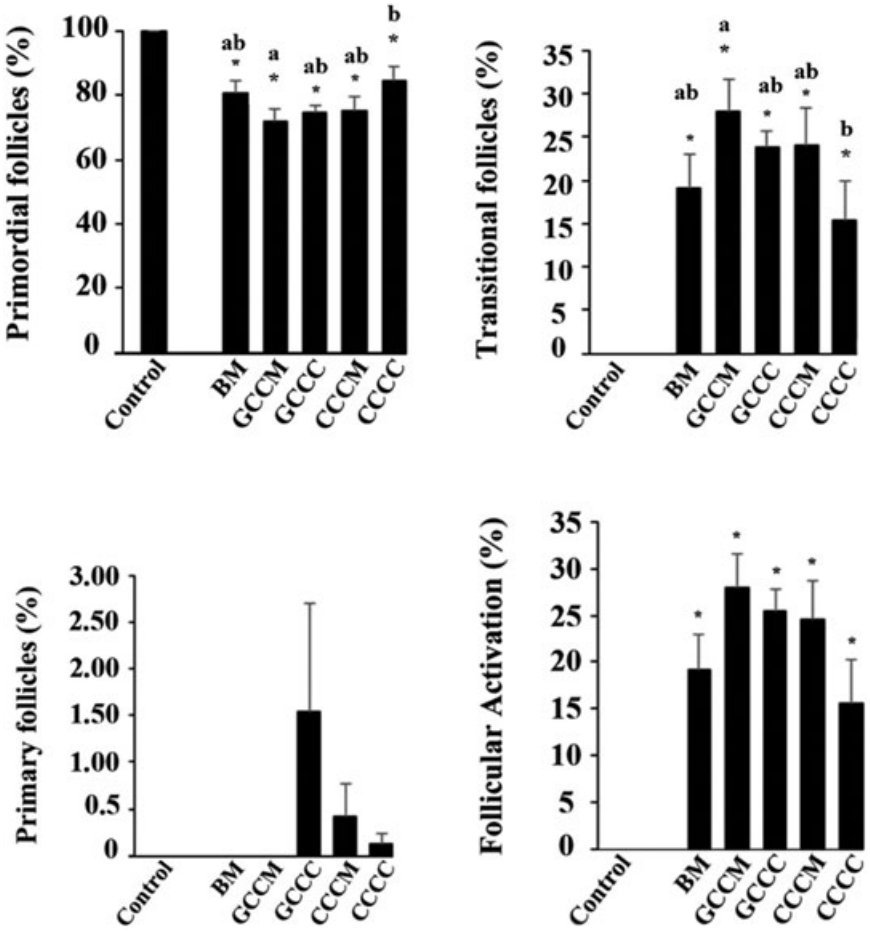

FIGURE 4 Proportions of the different follicle classes (a) and activation of primordial follicles before and after in vitro culture (b). *Significant difference in the specified group on Day 6 as compared with Day 1 ($p < 0.05$). Different letters indicate tendency for difference among experimental groups on Day 6 ($0.05 < p < 0.1$). BM: base medium; CCCC: cumulus cell coculture; CCCM: cumulus cell condition medium; GCCM: granulosa cell condition medium

culture and was not different among various experimental groups (Figure 4). Also, in all groups, the rate of follicular activation increased on Day 6 compared to fresh control ($p < 0.01$; Figure 4).

3.3 | Activators and inhibitors fluctuation in real-time PCR analysis

After in vitro culture, Pten expression in GCCM group was lower than that in BM group ($p = 0.020$); moreover, it tended to be lower in CCCM than BM group ($p = 0.090$; Figure 5). Compared with control, Pi3k expression only increased in CCCC group over the course of culture ($p = 0.032$); as a result, on Day 6, the Pi3k expression was higher in CCCC group than BM, GCCM, and CCCM groups ($p < 0.05$; Figure 5).

Cx37 expression increased over the 6-day period in the CCCM group ($p = 0.002$). On Day 6, the expression of CX37 was greater in the CCCM group compared with BM, GCCC and CCCC groups ($p < 0.01$; Figure 5).

Pi3k and Pten data were coupled to make an important item as activation index (Pi3k/Pten). Inhibition index (Pten/Pi3k) in GCCM and GCCC groups was lower than that in BM and CCCC groups

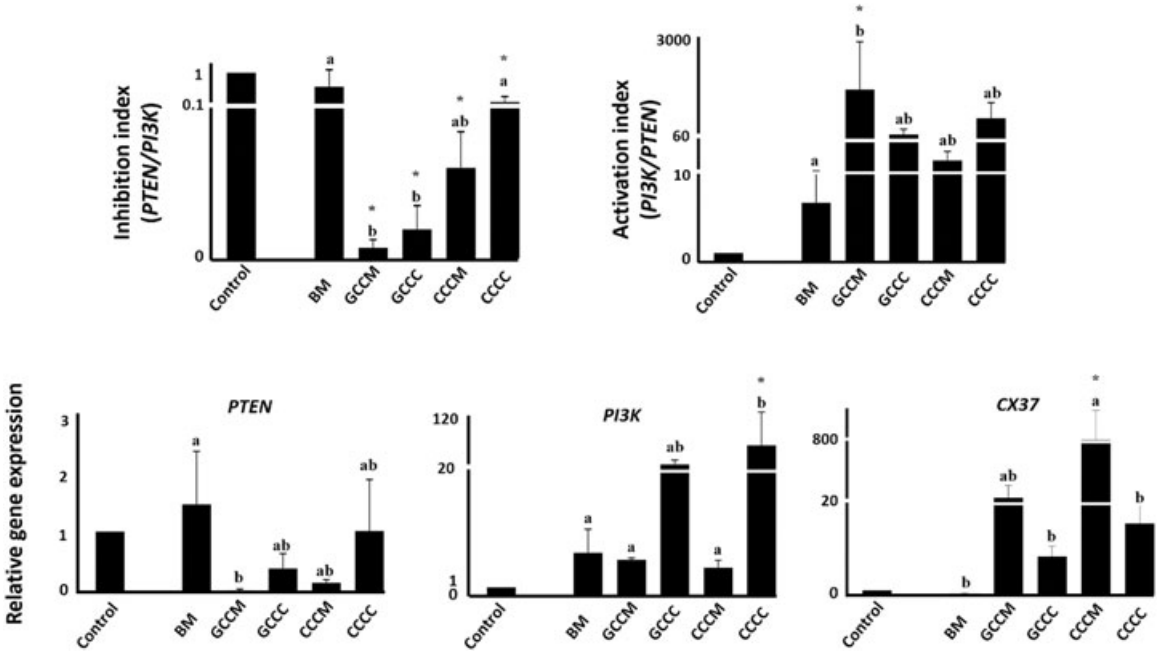

FIGURE 5 Gene expression of PTEN, PI3K, CX37 as well as inhibition and activation index on control and after in vitro culture. *Significant difference in the specified group on Day 6 compared with control ($p < 0.05$). Different letters indicate differences among experimental groups on Day 6 ($p < 0.05$). CX37: connexin 37; PI3K: phosphoinositide-3-kinase; PTEN: phosphatase and tensin homolog

($p < 0.05$). Additionally, activation index (Pi3k/Pten) was higher in GCCM than BM group ($p < 0.05$; Figure 5).

3.4 | Western blot analysis

PTEN protein decreased over time in GCCC group ($p = 0.029$) and tended to decline during culture in GCCM group ($p = 0.083$). However, PTEN protein did not significantly change over time in BM, CCCM and CCCC groups ($p > .05$). On Day 6, PTEN protein in GCCC group tended to be lower than that in BM ($p = 0.090$) and CCCC ($p = 0.052$) groups (Figure 6).

Phosphorylation of Akt decreased over culture in BM and CCCM groups ($p < 0.05$) but it did not significantly alter over time in GCCM, GCCC, and CCCC groups ($p > 0.05$). On Day 6, phosphorylation of Akt was greater in GCCM groups as compared with BM ($p = 0.007$) and CCCM ($p = 0.005$) groups.

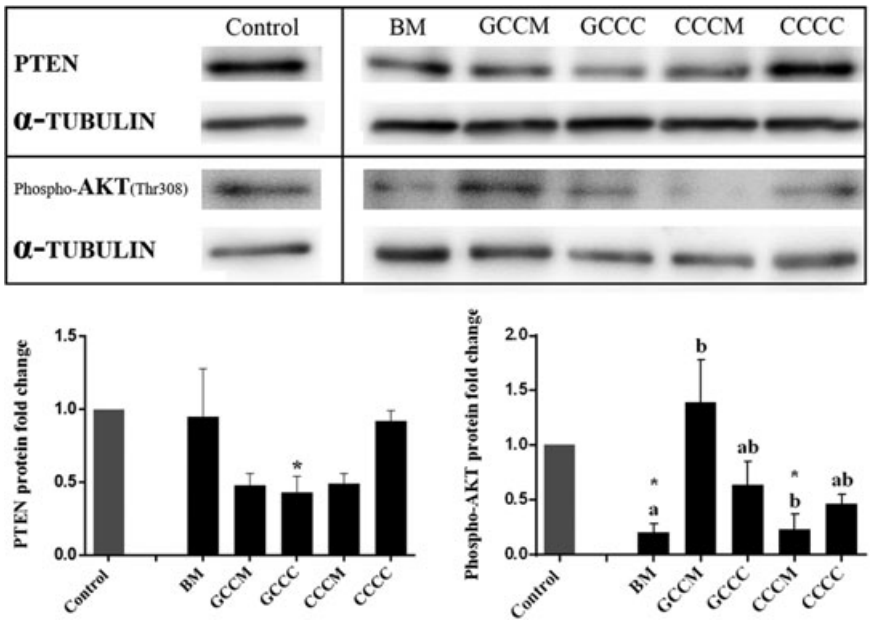

FIGURE 6 Level of PTEN and phosphorylated Akt evaluated using western blot analysis. *Significant difference in the specified group on Day 6 compared with control ($p < 0.05$). Different letters indicate differences among experimental groups on Day 6 ($p < 0.05$). Akt: protein kinase B; BM: base medium; CCCC: cumulus cell coculture; CCCM: cumulus cell condition medium; GCCM: granulosa cell condition medium; PTEN: phosphatase and tensin homolog

4 | DISCUSSION

Assessing the effect of coculture with granulosa and CCs, and the respective cells conditioned media on activation of primordial follicles during a 6-day in vitro culture, the current study revealed that the conditioned media of GCs and CCs and coculture with GCs could numerically enhance the rate of primordial activation in mice, while the results associated with coculture with CCs were not promising. In this regard, it was only the difference between GCCM and CCCC was statistically significant. CCs are the cells surrounding the oocyte after antrum formation and specialized to support maturation of oocyte before fertilization (Huang & Wells, 2010), whereas GCs, encompassing the oocyte before antral stage, are dedicated to facilitate transition of follicles from primordial to preantral stage (Z. Li et al., 2011; Skinner, 2005). Given the findings of the current study, it appears that GCs and their secreted factors could better support follicular activation under in vitro condition. GCs produce various growth factors including activin, epidermal growth factor (EGF), leukemia inhibitory factor, Kit L (Skinner, 2005; Zolti et al., 1991), basic fibroblast growth factor (Silva et al., 2004), colony stimulating factor 1 (Zolti et al., 1991), Insulin-like growth factor (Dirnfeld et al., 1997), EGF (Silva et al., 2004), transforming growth factor-β (Zolti et al., 1991), steroids (Fabbri et al., 2000), and cytokines, such as interleukin 1 and 6, all of which could contribute to primordial follicle activation.

Interestingly, PTEN mRNA or protein were lower in GCCM, GCCC, and CCCM groups as compared with BM and CCCC groups in the current study. PTEN is a phosphatase, which negatively regulates PI3K and catalyzes the conversion of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) to phosphatidylinositol 4,5-bisphosphate (PIP_2) via dephosphorylation of the 3-position of inositol ring in PIP3 (Brito, Goulielmaki, & Papakonstanti, 2015). As a result, PTEN is one of the main factors precluding follicular activation, thereby perpetuating the dormancy of primordial follicles (Kim, 2012). In this context, overactivation and early depletion of primordial follicles have been observed in oocyte-specific Pten deleted mice (Lan, Xu, & Cooney, 2004; Reddy et al., 2008). Moreover, addition of PTEN inhibitor to culture medium culminated in activation of dormant primordial follicles (J. Li et al., 2010; Novella-Maestre, Herraiz, Rodríguez-Iglesias, Díaz-García, & Pellicer, 2015). Therefore, the numerically accelerated activation of primordial follicles in the GCCM, GCCC, and CCCM groups could partly be attributed to downregulation of PTEN following in vitro culture.

In addition, phosphorylated Akt, as an indicator of PI3K enzymatic activity (Hemmings & Restuccia, 2012), was higher in GCCM than CCCM group, while the level of PTEN was quite comparable between these groups. As aforementioned, this phenomenon could have simply resulted from different molecular behavior of granulosa and CCs in spite of the fact that these cells are of the same origin (Latham, Bautista, Hirao, O'brien, & Eppig, 1999). For example, expression of KIT ligand, as an indispensable regulator of primordial follicle activation (John, Shidler, Besmer, & Castrillon, 2009; Jones & Pepling, 2013), declines as GCs differentiate to CCs (Hutt, McLaughlin, & Holland, 2006; Joyce, Pendola, Wigglesworth, & Eppig, 1999) and signal transduction of KIT ligand is mediated via PI3K/Akt pathway (John et al., 2009; Yao, Lau, & Ge, 2014). Nevertheless, such promotion of Akt phosphorylation by granulosa conditioned media was not observed in GCCC group, albeit PTEN was not different between GCCM and GCCC groups, which implicates that perhaps, presence of ovaries containing oocytes influenced molecular behavior of GCs since granulosa conditioned media resulted from GCs that had no contact with 1-day-old ovaries during culture, whereas in the GCCC group, GCs had direct contact with such ovaries. In this regard, growth differentiating factor 9 (GDF9), which originates from oocytes (Gilchrist et al., 2008; Otsuka, McTavish, & Shimasaki, 2011), is believed to inhibit KIT ligand secretion (Otsuka et al., 2011) as upregulation of KIT ligand has been observed in GCs of GDF9-null mice (Elvin, Clark, Wang, Wolfman, & Matzuk, 1999).

Although Pi3k expression was substantially augmented in CCCC group, this elevation at mRNA level was not reflected in the abundance of phosphorylated Akt. This observation besides high levels of PTEN mRNA and protein and low activation of primordial follicles in CCCC group imply that upregulation of PI3K without downregulation of PTEN could not lead to successful activation of primordial follicles due to the fact that PTEN is the upstream regulator of PI3K (Brito et al., 2015; Kim, 2012; Zhang et al., 2017; Zhao et al., 2017).

In addition, the application of conditioned media increased the expression of Cx37 and in case of CCs conditioned medium this increase was statistically significant. Cx37 belongs to the connexin family of gap junction proteins, which facilitate intercellular communication and contribute to cell differentiation, migration, and development (Kidder & Mhawi, 2002). In the ovary, CX37 is believed to play an essential role in intercellular communication between the oocyte and GCs, facilitating bidirectional signaling between the oocyte and the surrounding somatic cells, which is required for normal development of the follicle (Simon, Goodenough, Li, & Paul, 1997; Teilmann, 2005; Veitch et al., 2004). Teilmann (2005) demonstrated significant elevation in the number of CX37 gap junctions from primary to preantral ovarian follicles in mice. Using CX37-deficient mice, Carabatsos, Sellitto, Goodenough, and Albertini (2000) indicated that CX37 is essential for oocyte growth and acquisition of cytoplasmic meiotic competence in preantral follicles. Therefore, elevation in the expression of CX37 implicates the progression from primordial to primary stage in cultured ovaries and the potential of conditioned media to enhance in vitro follicular activation (Table 1).

In conclusion, the current study showed that GC conditioned medium enhanced activation of primordial follicles in 1-day-old mice ovaries under in vitro condition through suppressing the expression of Pten and stimulating PI3K activity. As a result, GC condition medium could be used if acceleration of in vitro activation of primordial follicles is desired. Nevertheless, it requires potential modifications to facilitate in vitro activation of primordial follicles beyond primary stage, which warrants further studies.

TABLE 1 Mouse primer sequences for real-time PCR

Official Symbol		Sequence	PCR product (bp)
Pten	Forward	TGGATTCGACTTAGACTTGACC	184
	Reverse	GCGGTGTCATAATGTCTCTCAG	
Pi3k	Forward	GGAGACCAGTCACCCTATC	213
	Reverse	CCGAGGTAGTGAGAGGATGTC	
Cx37	Forward	GTTCCTCTTCGTCAGCAC	254
	Reverse	CCAGCACACTCTTACACAG	

Note. CX37: connexin 37; PCR: polymerase chain reaction; PI3K: phosphoinositide-3-kinase; PTEN: phosphatase and tensin homolog.

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