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Expression of genes involved in the non-neuronal cholinergic system and their possible functions during ovarian follicular development in mice

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Abstract: The regulatory roles of the non-neuronal cholinergic system, such as its growth promoting effects on mural granulosa cells during ovarian folliculogenesis, have been reported for several mammalian species; however, its roles in the early stages of follicles are not well-understood. This study was conducted to examine the expression of transcripts involved in the non-neuronal cholinergic system, and the effects of activating acetylcholine (ACh) signaling in mouse ovaries. The transcripts encoding proteins required in processes critical for ACh metabolism and a nicotinic cholinergic receptor, CHRNA7, were detected in ovaries and isolated mural granulosa cells. Stimulation with carbachol, a cholinergic agonist, promoted proliferation of mural granulosa cells *in vitro*. Moreover, carbachol treatment of neonatal ovaries significantly increased the numbers of primordial follicles compared with control untreated ovaries in organ cultures. These results suggest that a functional non-neuronal cholinergic system exists in mouse ovaries, and that it promotes the proliferation of mural granulosa cells and the formation/survival of primordial follicles.

Key words: Ovary, Granulosa cells, Acetylcholine, Mouse

Introduction

In mammals, a pool of dormant primordial follicles comprises the ovarian reserve, which determines the reproductive potential of female animals [1]. Therefore, the formation and maintenance of the ovarian reserve is a critical step determining the fertility of female animals; however, the mechanisms regulating this process are still largely unknown.

While acetylcholine (ACh) is known as an important neurotransmitter in both the central and peripheral nervous systems, its production has been detected in many non-neuronal cells and tissues, including the ovaries of some mammalian species [2]. The production of ACh has been reported in granulosa cells of antral follicles in humans and rats, and luteal cells in cattle [3, 4]. Stimulation with carbachol, a cholinergic agonist, promoted the proliferation of human granulosa cells [5], and ACh chloride treatment prevented apoptosis of bovine luteal cells [4]. Moreover, *in vivo* blockade of acetylcholinesterase (ACHE), an enzyme which hydrolyses ACh, resulted in increased ACh levels, and enhanced follicular development in rat ovaries [6]. Therefore, it is likely that the non-neuronal cholinergic system exists in ovaries, and non-neuronal cell-derived ACh possesses trophic, growth promoting effects on granulosa/luteal cells, at least, in some mammalian species [4, 7]. However, its role in regulating the early stages of follicular development is not well understood.

The cholinergic system, in general, involves the following processes. The precursor choline is incorporated into cells through several different transporters such as a high-affinity choline transporter (CHT1) (also known as SLC5A7) and choline transporter-like proteins (CLTs) (also known as SLC44A1-3). The synthesis of ACh requires an enzyme, either choline acetyltransferase (CHAT) or carnitine acetyltransferase (CRAT, also known as CarAT) [8–10]. Then, the synthesized ACh is released from cells through several different mechanisms which

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involve vesicular ACh transporter (VAChT) (also known as SLC18A3), organic cation transporters (OCTs) (also known as SLC22A1-3), CLT4 (also known as SLC44A4), and mediatophore/ATP6V0C [11, 12]. After its release, ACh binds to muscarinic cholinergic receptors (CHRMs) and nicotinic cholinergic receptors (CHRNs), or is immediately degraded by acetylcholinesterase (ACHE) and butyrylcholinesterase (BCHE). The specific expressions and requirements of these enzymes and transporters in ovarian ACh metabolism, however, have not been fully characterized.

This study aimed to examine the roles of the non-neuronal cholinergic system in the follicular development in mice. Since the presence of the non-neuronal cholinergic system has never been assessed in mouse ovaries in detail, we first examined the expression of transcripts involved in the non-neuronal cholinergic system in the ovaries and isolated mural granulosa cells. Then, the effects of carbachol on the proliferation of mural granulosa cells and the early stages of follicular development were examined *in vitro*.

Materials and Methods

Mice

(C57BL/6×DBA2) F1 (BDF1) female mice were purchased from Sankyo Labo Service Corporation (Tokyo, Japan), or produced and raised in the research colony of the authors at The University of Tokyo. All the experiments were approved by the Animal Care and Use Committees of The University of Tokyo.

Culture medium

The basic culture medium used was bicarbonate buffered MEMα (Thermo Fisher Scientific, Gaithersburg, MD) supplemented with 75 *μ*g/ml penicillin G, 50 *μ*g/ml streptomycin sulfate, and 0.23 mM pyruvate. For culturing mural granulosa cells, 10% fetal bovine serum (FBS) was added to the basic culture medium (MEMα + FBS). The medium used for organ culture of neonatal ovaries (organ culture medium) was the basic culture medium supplemented with 5% FBS and 1% ITS (Thermo Fisher Scientific).

Isolation and culture of mural granulosa cells

Mural granulosa cells were isolated from 3-week-old female mice injected with pregnant mare serum gonadotropin (PMSG)(ASKA Pharmaceutical, Tokyo, Japan) 42–46 h prior to each experiment as reported previously [13] and stored at −80 °C in a lysis buffer provided with a ReliaPrep RNA Miniprep system (Promega, Tokyo, Japan) (described below).

In some experiments, mural granulosa cells were centrifuged, re-suspended in MEMα + FBS medium, and filtered through a 70-*μ*m cell strainer (Falcon, Tokyo, Japan). 5×10^3 cells were transferred to individual wells of a 96-well plate (IWAKI, Tokyo, Japan). After 3 h of culture, the cells were washed 3 times with PBS and cultured for an additional 24 h in MEMα+FBS medium with or without carbachol supplementation (0–100 *μ*M, Wako Pure Chemical Industries, Osaka, Japan). After the culture, cell proliferation assays were performed using Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to the manufacturer's instruction. Cultures were conducted at 37 °C in 5% O_2, 5% CO_2, and 90% N_2.

Isolation of organs, total RNA extraction, and reverse-transcription polymerase chain reaction (RT-PCR)

To compare transcript levels among different organs, ovaries, testes, brains, spinal cords, kidneys, and adrenal glands were isolated from 3-week-old BDF1 mice. Total RNA was isolated from these organs and mural granulosa cells of 3-week-old PMSG-primed mice using ReliaPrep RNA Miniprep system (Promega) according to the manufacturer's instruction. Complementary DNA was prepared from 1 *μ*g of total RNA by oligo-dT-primed polymerization using SuperScript III reverse transcriptase (Invitrogen, Tokyo, Japan) according to the manufacturer's instruction.

PCR reactions were performed using BioTaq DNA polymerase (Bioline) according to the manufacturer's instruction. The sequences of the PCR primers, annealing temperatures, and numbers of amplification cycles for each transcript are summarized in Table 1. The PCR products were separated by agarose gel electrophoresis, and the DNA bands were visualized by ethidium bromide staining.

Immunoblot

Immunoblot analysis was conducted as previously reported [14] using anti-Cyclin D2 monoclonal antibody (DCS-5, Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) and anti-*β*-actin polyclonal antibody (GTX109639, GeneTex, Irvine, CA). The secondary antibodies used were peroxidase-conjugated goat anti-rabbit IgG (AP132P, Millipore) or goat anti-mouse IgG + IgM (115-035-044, Jackson ImmunoResearch Laboratories, West Grove, PA). The signals were visualized with an ImmunoStar LD western blotting detection reagent (Wako Pure Chemicals, Osaka, Japan), C-DiGit Blot Scanner and Image Studio software for C-DiGit (LI-COR, Nebraska, USA) according to the manufacturers' instructions.

Table 1. Primer sets used for RT-PCR

Gene	Accession no.	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')	Amplicon size (bp)	annealing tempera-tures (°C)	Cycles
Slc5a7	NM_022025	GTGGTCTAGCTTGGGCTCAG	AGCTGCCAGGAAGGACAGTA	596	50	35
Slc44a1	NM_133891	GAATGGAAGCCATTGGAAGA	TGTCGAGGACATGCTGCTAC	311	50	30
Slc44a2	NM_152808	CTTAGCCATCGTGCAGATCA	CATGCCATAGACGCTGAAGA	454	50	30
Slc44a3	NM_145394	ATAAGCCTCTTTCCGGCATT	CTGTCCCGTTAATGGCAGTT	408	50	35
Chat	NM_009891	CCTGCCAGTCAACTCTAGCC	ACGAGCTTCTTGTTGCCTGT	780	50	35
Crat	NM_007760	CAAGCAGGACTCAGTGGTGA	GGGCTTAGCTTCTCGGACTT	732	50	30
Slc44a4	NM_023557	ACCAAGTGTTTCGGGACAAG	TAGCCCTTGAAGACGCACTT	445	50	35
Slc22a1	NM_009202	GTAAGCTCTGCCTCCTGGTG	GACCATCTGCAACACAATGG	824	50	30
Slc22a2	NM_013667	CTCAGCCAGTGCATGAGGTA	CTCCGGTATGCACCAGAAAT	600	50	35
Slc22a3	NM_011395	ATCCTGAACCTTGGCTTCCT	CCTCATTTGGGGAGTTCTCA	567	50	35
Slc18a3	NM_021712	CCCTTAGTGGTCTCGCTCTG	GGAGGCACCAGGGTGTAGTA	811	50	30
Atp6v0c	NM_009729	GCTTGTCTTTTCCCTGCTTG	TCCTGTGACTGATGGTGGAA	518	50	30
Ache	NM_009599	TCTTCCGATTTTCCTTCGTG	TGAGCAATTTGGGGAGAAAG	741	50	35
Bche	NM_009738	TGGCTTACCTCTGGGAAGAA	TTGATTTTGCCAGTCCATCA	343	50	35
Chrm1	NM_001112697	TCCCATGGAAACCCTGAATC	TTCATGACAGAGGCGTTGCT	514	50	35
Chrm2	NM_203491	CACTGGGAGAAGTGGAGGAG	GTGGTCCGCTTAACTGGGTA	745	55	35
Chrm3	XM_006516468	TGACCAGCAATGGCATCTAC	AGGCCAGGCTTAAGAGGAAG	570	50	35
Chrm4	NM_007699	CGGAGCAAGACAGAAGCAG	GCAGAAATAGCGGTCAAAGC	450	55	35
Chrna1	NM_007389	AGTCACCGTGGGTCTACAGC	CTGCAGTTCTGCTCATCGAA	339	50	35
Chrna2	M_144803	AGCCAGCTCCTTGTAGTCCA	GTAGGTGACGTCGGGGTAGA	747	50	35
Chrna3	NM_145129	CTACCAAGGGGTGGAGTTCA	GGCAGGTAGAAGACGAGCAC	492	55	35
Chrna4	NM_015730	TCGTCTAGAGCCCGTTCTGT	GGCGTAGGTGATGTCAGGAT	739	50	35
Chrna5	NM_176844	AAAAGTGGGTTCGTCCTGTG	CGCTCATGATTTCCCATTCT	492	55	35
Chrna6	NM_021369	AACCTGCACTCCGGTTTATG	GGGCAACCTCCTGATGTAGA	696	50	35
Chrna7	NM_007390	GCAGATCATGGATGTGGATG	GAGCACACAAGGAATGAGCA	550	55	35
Chrna9	NM_001081104	CACGCTCTCCCAGATAAAGG	AACCAAAGGTCAGGTTGCAC	338	50	35
Chrna10	NM_001081424	CTTGAGACCAGTGGCAGACA	GAACACACAGGGCAGCAGTA	616	50	35
Actb	NM_007393	GCACCACACCTTCTACAATG	TGCTTGCTGATCCACATCTG	822	50	26

Band intensities were analyzed using ImageJ software (US National Institutes of Health).

Total RNA extraction, reverse transcription, and real-time PCR

Total RNAs were isolated from ovaries using a ReliaPrep RNA Miniprep system (Promega, Tokyo, Japan) according to the manufacturer's instruction. The extracted RNA was then reverse-transcribed using a ReverTra Ace qPCR Master Mix with gDNA Remover kit (TOYOBO, Osaka, Japan) according to the manufacturer's instruction.

The steady-state levels of transcripts were determined by real-time PCR using ThunderbirdTM SYBR® qPCR Mix (TOYOBO) with a StepOnePlusTM Real-Time PCR System (Applied Biosystems) as reported previously [13]. The PCR primer sets used were as follows: 5′-CTACAATACGTCCGGCTCT-3′ and 5′-CGGTCCAGGTTAAACAGCAG-3′ for *Gdf9*; 5′-AGCAGGCCCTGTTAGTGCTAT-3′ and 5′-CGAGTAGGGCAGAGGTTCTGT-3′ for *Bmp15*; and 5′-ATCTGGGCATTGCCAGTATC-3′ and 5′-AGTTTGCACTGCTGCACATC-3′ for *Chrna7*. The primer set for *Rpl19* amplification was reported previously [15]. The results are presented as the expression levels relative to the transcript amount of postnatal day 0 (PD0) ovaries after normalization to the expression levels of a housekeeping transcript, *Rpl19*, by the $2^{-\Delta\Delta Ct}$ method [16]. The reactions were run in duplicate and melting curve analyses were performed at the end of the reaction to avoid false-positive signals. The PCR products were subjected to agarose gel electrophoresis to confirm their sizes.

Organ culture of neonatal mouse ovaries

PD0 ovaries were rinsed with a mixture containing 50% (vol/vol) of the organ culture medium and Matrigel (Corning Matrigel, Corning), then transferred into a 10-*μ*l drop of the Matrigel/medium mixture placed on a cell culture insert (Falcon, Tokyo, Japan). Then, the culture inserts were set into wells of a 24-well plate (IWAKI) and incu-

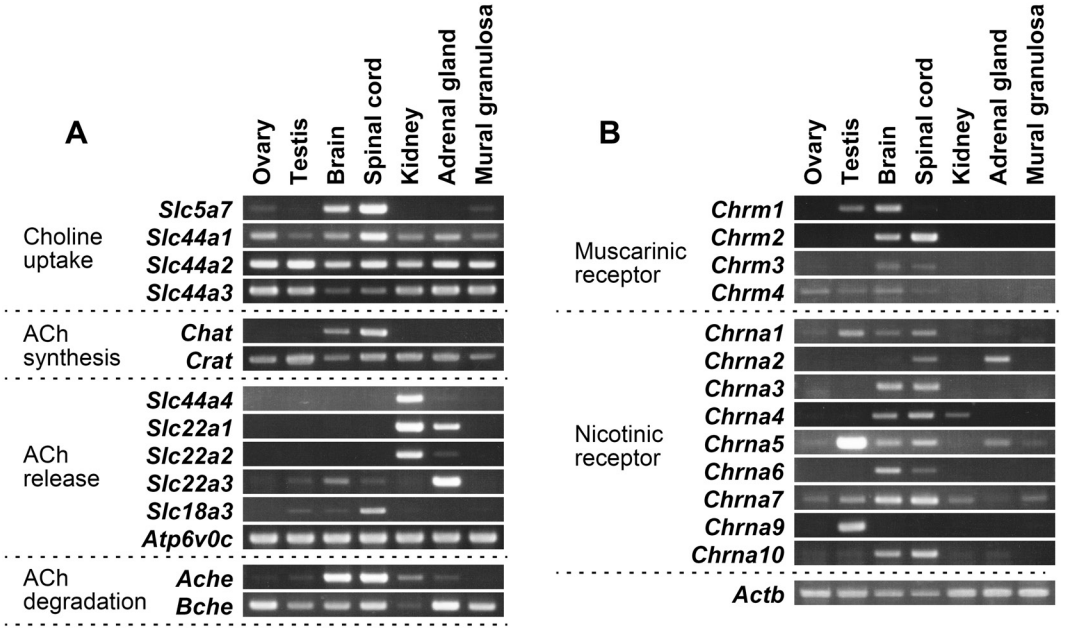

Fig. 1. Detection of transcripts involved in ACh metabolism and ACh receptors in mouse ovaries. Expression of transcripts involved in ACh metabolism (A) and its receptors (B) were compared among different mouse organs and isolated mural granulosa cells.

bated at 37 °C to solidify the Matrigel/medium mixture. After Matrigel had solidified, 500 μl of the organ culture medium with or without 10 μM of carbachol supplementation were added to each well of the 24-well plate (10 μM). Cultures were conducted for 48 h at 37 °C under 5% CO_2 in air.

Histological assessment of ovaries

Ovaries were fixed in Bouin's fixative for paraffin sectioning. Paraffin sections of 6 μm thickness were stained with hematoxylin and eosin, dehydrated and embedded in Permount (Fisher Scientific, Pittsburgh, PA).

Follicular counts were conducted on the largest section of each ovary. The criteria used for classifying the developmental stages of follicles were as follows: unassembled follicles, clusters of oocytes without any definite somatic cells; primordial follicles, oocytes surrounded by a single squamous layer of follicular somatic cells; and primary follicles, oocytes surrounded by a single layer of cuboidal granulosa cells [17, 18].

Statistical analysis

Statistical analyses were conducted using Microsoft Excel (Microsoft) with add-in Excel-statistics software (Social Survey Research Information Co, Ltd.). The Tukey test or the standard *t*-test was used for multiple or paired comparisons, respectively. A P value <0.05 was considered to be statistically significant.

Results

Detection of transcripts involved in ACh metabolism and ACh receptors in ovaries

Expression of transcripts involved in ACh metabolism and its receptors were examined in the ovary and isolated mural granulosa cells, and compared with other organs (i.e., testis, brain, spinal cord, kidney, and adrenal gland). These organs were selected based on their relatively high expressions of ACh-related transcripts indicated in the publicly available BioGPS database (http://biogps.org/) [19].

Transcripts encoding proteins required for choline uptake (*Slc44a1-3*), ACh synthesis (*Crat*), ACh release (*Atp6v0c*), and ACh degradation (*Bche*) were detected in boththe ovary and isolated mural granulosa cells (Fig. 1A). A low level of *Chrm4* expression was detected in the ovary, however, it was barely detectable in isolated mural granulosa cells (Fig. 1B). The only ACh receptor transcript detectable in mural granulosa cells was *Chrna7*. Other ACh receptors were detectable in neither the ovary nor mural granulosa cells under the present experimental conditions. Therefore, the transcripts encoding proteins

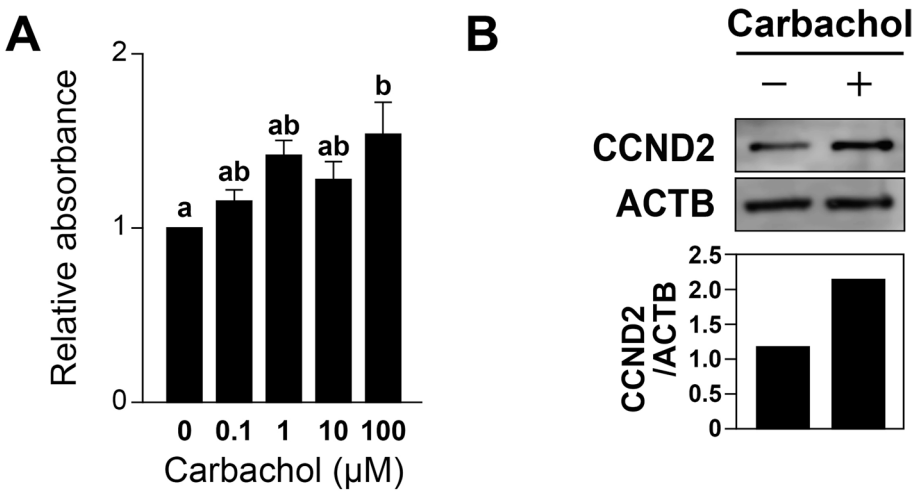

Fig. 2. Effects of carbachol on proliferation of mural granulosa cells. (A) Mural granulosa cells were treated with carbachol and viable cell numbers were evaluated after 24 h of treatment. Values are shown as the mean±SEM of 3 repeats of independent experiments. Values denoted by different letters (a and b) are significantly different ($P<0.05$). (B) Expression levels of cyclin D2 (CCND2) were evaluated by western blotting (n=2).

required in processes critical for ACh metabolism and an ACh receptor, *Chrna7*, were expressed in the ovary and mural granulosa cells.

Effects of carbachol on proliferation of mural granulosa cells

It has been reported that activation of ACh signaling using a cholinergic agonist, carbachol, promoted the proliferation of human granulosa cells [5]; however, the effect of ACh on proliferation of granulosa cells in other mammalian species was not previously known. Therefore, the effect of carbachol treatment on the proliferation of mouse granulosa cells was examined. Mural granulosa cells isolated from PMSG-primed antral follicles were treated with carbachol, and the number of viable cells was evaluated after 24 h of the treatment. In addition, since the proliferation of granulosa cells requires Cyclin D2 (CCND2) expression [20], changes in the protein levels of CCND2 responding to the carbachol treatment were examined.

As shown in Fig. 2A, the carbachol treatment dose dependently increased viable cell numbers and the increase became significant at a concentration of 100 μM carbachol, compared to a non-treatment group (Fig. 2A). Moreover, CCND2 protein level was higher in mural granulosa cells treated with carbachol than in those not treated (Fig. 2B). These results strongly suggest that mural granulosa cells are capable of responding to ACh stimulus, and that activating the ACh signal promotes proliferation of mural granulosa cells in mice, as was reported for human granulosa cells.

Assessment of Chrna7 expression during ovarian development

Since *Chrna7* was the only ACh receptor detected in granulosa cells under the present experimental conditions, the expression kinetics of *Chrna7* transcript during ovarian development was examined in detail using real-time PCR (Fig. 3). Moreover, transcripts encoding growth differentiation factor 9 (GDF9) and anti-Mullerian hormone (AMH) were also examined as markers of follicular development. *Gdf9* and *Amh* mRNAs are expressed in oocytes and granulosa cells, respectively, starting from primary follicles [21, 22].

As shown in Fig. 3, the expression levels of *Gdf9*, *Amh* and *Chrna7* transcripts were low in PD0 ovaries, but gradually increased with ovarian development. While the high expression levels of *Gdf9* and *Amh* were maintained in the ovaries of PD12 mice, the *Chrna7* expression level was significantly decreased compared to PD4 ovaries.

Effects of carbachol on the early stages of follicular development

The high expression levels of *Chrna7* transcripts in neonatal ovaries (PD0–4, Fig. 3) prompted us to test the effects of ACh on the development of early follicles. To do this, ovaries of PD0 mice were organ cultured with or without carbachol supplementation, and the dynamics

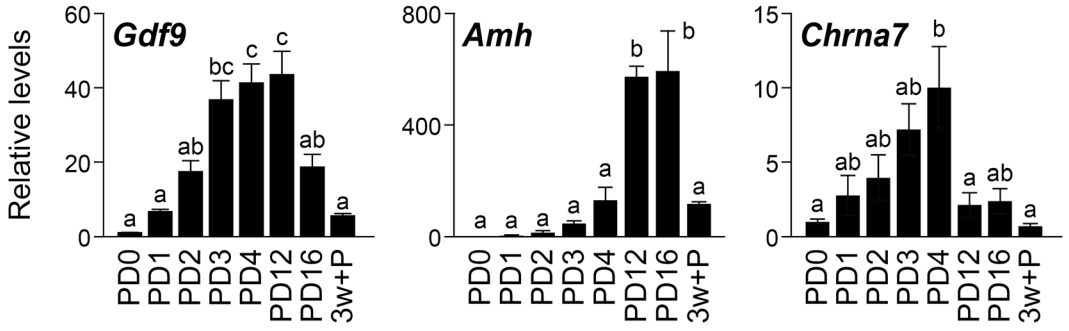

Fig. 3. Expression dynamics of *Chrna7* during ovarian development. Expression levels of *Gdf9*, *Amh*, and *Chrna7* were examined in ovaries of postnatal day 0 to 16 (PD0–16) and PMSG-primed 3-week-old (3w+P) mice. Values are shown as the mean ±SEM of more than 4 repeats of independent experiments. Values denoted by different letters (a, b and c) are significantly different ($P<0.05$).

of follicular development was assessed after 48 h of culture. In addition, the expression levels of the *Gdf9*, *Amh*, and *Chrna7* transcripts were also examined.

The ovaries of the PD0 mice predominantly consisted of unassembled follicles. which were identified as clusters of oocytes without any definite somatic cells (not shown). After 48 h of culture, significant reductions in the numbers of unassembled follicles and the formation of primordial follicles were observed in both control and carbachol-treated ovaries (Fig. 4A and B). The numbers of primordial follicles were significantly higher in carbachol-treated ovaries than in control ovaries (Fig. 4B). The number of primary follicles tended to be higher in carbachol-treated ovaries than in the control, but the difference was not statistically significant. Moreover, expression levels of *Gdf9*, *Amh*, and *Chrna7* were not significantly different between the two groups (Fig. 4C).

Discussion

The trophic, growth promoting effect of the non-neuronal cholinergic system on ovarian granulosa/luteal cells has been reported for several mammalian species. The present results show that ACh-related transcripts are expressed in mouse mural granulosa cells, and that activating the ACh stimuli promoted the proliferation of mural granulosa cells and the development of early follicles *in vitro*. Therefore, the non-neuronal cholinergic system seems to exist and play a trophic, growth promoting roles during follicular development in mouse ovaries. Since the roles of the non-neuronal cholinergic system during the early stages of follicular development have never previously been reported, the present results are the first to describe its roles during the period in mammals.

Although the growth promoting effect of ovarian ACh signaling appears to be conserved, the receptors mediating this ACh effect may be different among mammalian species. In humans, carbachol treatment promoted proliferation of granulosa cells, and this effect of carbachol was prevented by a selective muscarinic receptor antagonist, pirenzepine [5]. This indicates that the growth promoting effect of the ACh signal in human granulosa cells is mediated by muscarinic cholinergic receptors (CHRMs). The expression of CHRMs has also been reported in the ovaries of rhesus monkeys and rats [3, 23]. On the other hand, in the present study, the only ACh receptor detected in mouse mural granulosa cells was a nicotinic cholinergic receptor, CHRNA7. Whether or not the growth promoting effect of carbachol on mouse granulosa cells is mediated by CHRNA7 needs further investigation. However, since the expression of CHRMs was not detected in mouse mural granulosa cells in this study, and the association of CHRNs with cell proliferation has been reported in several cell types [24–26], it is possible that CHRNA7 mediates the growth promoting effects of the ACh signal in mouse mural granulosa cells.

In addition to the receptor expressions, there may be species-dependent variations in the transcripts involved in ACh metabolism in mammalian ovaries. While two acetyltransferases, namely CHAT and CRAT, are known to be able to synthesize ACh [8–10], CHAT seems to be a major acetyltransferase in granulosa cells of humans, rhesus monkeys, rats, and cattle [4, 5, 27]. In the present study, however, *Chat* transcripts were undetectable in mouse granulosa cells, whereas *Crat* transcripts were readily detected. Interestingly, Fritz and colleagues reported that, while human granulosa cells were stained positive by immunohistochemical staining using CHAT

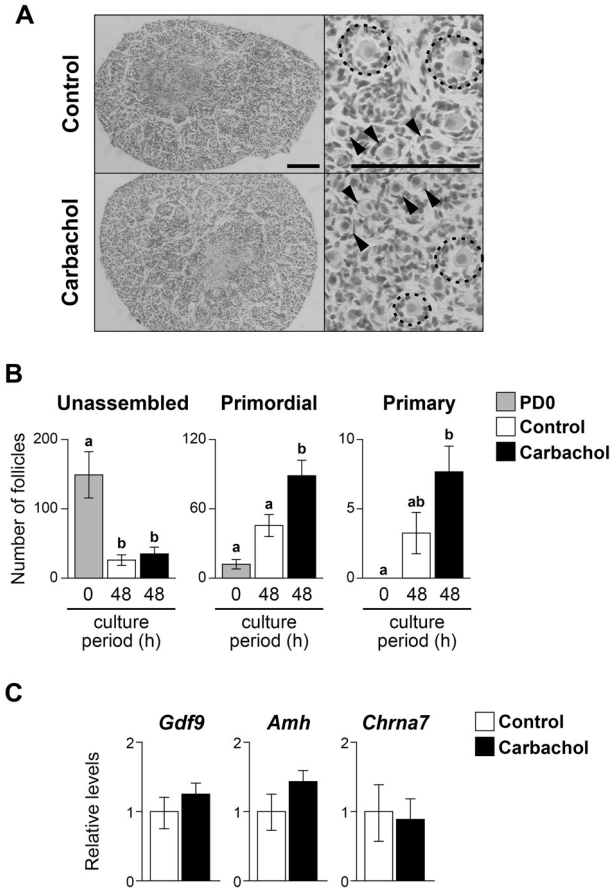

Fig. 4. Effects of carbachol on early stages of follicular development. (A) Representative photographs of PD0 ovaries after 48 h of organ culture with (bottom) or without (top) carbachol treatment ("carbachol" and "control", respectively). Bar = 100 μm. (B) Follicular count of freshly isolated PD0 ovaries, and organ cultured control- and carbachol-treated ovaries. Follicular counts were conducted for unassembled, primordial and primary follicles using at least three ovaries for each treatment. Values denoted by different letters (a and b) are significantly different (P<0.05). (C) Expression levels of *Gdf9*, *Amh*, and *Chrna7* were examined in ovaries after 48 h of organ culture with or without carbachol treatment. Values are shown as the mean±SEM of more than 3 repeats of independent experiments.

antibody, *Chat* transcripts were not, rather *Crat* transcripts were detected instead [3]. This suggests the possibility of cross-reactivity of the antibody between the two acetyltransferases which have relatively high homology in amino acid sequences [3]. Whether this is the case in other mammals remain to be elucidated. Likewise, of the two cholinesterases, ACHE and BCHE, the expression of ACHE was reported in granulosa cells of rhesus monkeys and rats [6, 7]; but, while both ACHE and BCHE were detected in human follicular fluid, only ACHE activity was detected in human granulosa cells [7]. In contrast, the only transcript encoding cholinesterase detected in mouse granulosa cells in this study was *Bche*. Whether or not these species-dependent variations also exist in proteins involved in choline uptake and ACh release needs further investigations.

The present results show that carbachol treatment of organ cultures increased the number of primordial follicles in mouse neonatal ovaries. Since a progressive loss of primordial follicles due to germ/somatic cell apoptosis (i.e., follicular atresia) occurs in developing ovaries [28–30], and ACh receptor signals have been shown to confer resistance to apoptosis on many cell types [31], the increase in the number of primordial follicles in carbachol-treated ovaries may be attributable to a reduction in follicular loss through inhibition of apoptosis. It is important to note that, in contrast to the present results, an increase in ovarian ACh content increased the number of small secondary follicles in rats; however, it had no effects on the numbers of primordial or primary follicles [6]. Therefore, although ACh commonly exerts trophic, growth promoting effects on follicles in both mice and rats, the particular stage of follicular development in which ACh actually exerts its function may differ between mice and rats. Although the underlying mechanisms need further investigation; it is possible that species-dependent variations in ACh receptor expression may be one of the reasons for the observed differences.

Although the results of the present study suggest that a functional non-neuronal cholinergic system exists in mouse ovaries, a number of questions remain to be answered. First, is the amount of ACh produced within a follicle sufficient to exert the effects observed in the present study? And, are the observed effects mediated by CHRNA7 or other unknown receptors? Moreover, the source of ACh that affects early follicular development *in vivo* has yet to be determined. Further studies investigating these uncertainties will be crucial for developing an understanding of the roles of ACh during follicular development in mammals.

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