Vasopressin Promoter Transgenic and Vasopressin Gene-Edited Ascidian, *Ciona intestinalis* Type A (*Ciona robusta*): Innervation, Gene Expression Profiles, and Phenotypes

Tsuyoshi Kawada¹, Akira Shiraishi¹, Shin Matsubara¹, Akiko Hozumi², Takeo Horie², Yasunori Sasakura² and Honoo Satake¹*

¹ Bioorganic Research Institute, Suntory Foundation for Life Sciences, Kyoto, Japan, ² Shimoda Marine Research Center, University of Tsukuba, Shimoda, Japan

Oxytocin (OT) and vasopressin (VP) superfamily neuropeptides are distributed in not only vertebrates but also diverse invertebrates. However, no VPergic innervation of invertebrates has ever been documented. In the ascidian, *Ciona intestinalis* Type A (*Ciona robusta*), an OT/VP superfamily peptide was identified, and the *Ciona* vasopressin (CiVP) induces oocyte maturation and ovulation. In the present study, we characterize the innervation and phenotypes of genetically modified *Ciona*: CiVP promoter-Venus transgenic and CiVP mutants. CiVP promoter-Venus transgenic *Ciona* demonstrated that CiVP gene was highly expressed in the cerebral ganglion and several nerves. Fluorescence was also detected in the ovary of young CiVP promoter-Venus transgenic ascidians, suggesting that the CiVP gene is also expressed temporarily in the ovary of young ascidians. Furthermore, a marked decrease of post-vitellogenic (stage III) follicles was observed in the ovary of CiVP mutants, whereas pre-vitellogenic (stage I) and vitellogenic (stage II) follicles were increased in the mutant ovary, compared with that of wildtype *Ciona*. Gene expression profiles showed that the expression of various genes, including genes related to ovarian follicle growth, was altered in the ovary of CiVP mutants. Altogether, these results indicated that CiVP, mainly as a neuropeptide, plays pivotal roles in diverse biological functions, including growth of early-stage ovarian follicles via regulation of the expression of a wide variety of genes. This is the first report describing a VP gene promoter-transgenic and VP gene-edited invertebrate and also on its gene expression profiles and phenotypes.

Keywords: ascidian, *Ciona intestinalis*, oxytocin, vasopressin, ovary, transgenic, gene edition
INTRODUCTION

Oxytocin (OT) and vasopressin (VP) superfamily peptides are one of the most widely distributed neuropeptides and/or neurohypophysial hormones in both vertebrates and invertebrates (1–10). These peptides are typically comprised of 9 to 14 amino acids, and share Cys1 and Cys6, which form an intramolecular disulfide bridge essential for biological activities. Moreover, the vertebrate OT family peptides share a neutral aliphatic amino acid (Leu, Ile, Val or Thr), whereas the vertebrate VP family peptides contain a basic one (Lys or Arg) at position 8. These superfamily peptides play pleiotropic roles in reproductive behaviors, osmoregulation, food intake, learning, and social behavior and are implicated with pathological processes such as anxiety and autism (1, 2, 11–13). Molecular phylogenetic trees demonstrated that the OT and VP superfamily have been diverged via duplication of a common ancestral gene during the process of agnathan to gnathostome evolution (1, 2, 4, 9–12).

OT/VP superfamily peptides have been identified in most invertebrates including nematodes, mollusks, annelids, insects, echinoderms, amphioxus, and ascidians (1–10, 14–16). Invertebrate OT/VP peptides also manifest some unique biological effects: induction of egg-laying behavior in earthworm (2, 17, 18); induction of unusual extra-oral feeding behavior in starfish (19); indirect induction of diuretic activity in beetles via stimulation of Malpighian tubules (5); modulation of gustatory associative learning in nematodes via effects of salt chemotaxis (8); modulation of parental-offspring social behavior via food-leaving in nematodes (20).

Recently, we identified unique biological roles of the VP/OT peptide of the cosmopolitan species of ascidians, *Ciona intestinalis* Type A (*Ciona robusta*). The *Ciona* VP peptide, CiVP (3) triggers oocyte maturation via germinal vesicle breakdown and ovulation (21). However, the CiVergic transcriptomes between the wildtype and the genome-edited transgenic larvae, young adults, and adults, and comparative transcriptomes between the wildtype and the genome-edited CiVP-knockout *Ciona*.

MATERIALS AND METHODS

Animals

Wildtype *Ciona* were cultivated and collected at Onagawa Bay, Maizuru Bay, Sagami Bay, Tosa Bay, and Mukaishima, Ascidians were cultured using an inland system described by Joly et al. (22).

PCR Primers

All PCR primers were ordered from Thermo Fisher Scientific Japan (Yokohama, Japan) or SIGMA ALDRICH JAPAN (Tokyo, Japan).

Plasmid Construction for Generation of Transgenic Ascidians

5’-untranslated region (UTR) sequence of CiVP gene was obtained from Ghost Database (http://ghost.zool.kyoto-u.ac.jp/blast_kh.html). *Ciona* genomic was prepared from sperms using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA). An approximately 5-kb segment of *GVP* gene 5’-UTR was amplified using the *Ciona* genomic DNA and gene-specific primers with a Sal I site (gattaGTCGACAttaaggaagcgcgctc, gattaGTCGACatgattaaactgtgtacat), and a thermal cycler GeneAmp PCR system 9700 (Thermo Fisher Scientific, Waltham, MA, USA). The sequence of the 5-kb segment was shown in **Supplemental Figure 1**. The 5-kb segment was inserted into a Sal I site of pSP-Venus vector, followed by two steps of amplification of the segment encoding CiVP 5’-UTR and Venus coding region. The first PCR was performed using the pSP-CiVP 5’-UTR-Venus plasmid with specific primers containing partial sequences of attB (AAAAAGCGG GCTAAATGAGAATTCGGCTCGTCCCT, AGAAGGCTGTTACTGTGACGCTCGAT). The PCR program was 98°C for 30 sec, 10 cycles of 94°C for 15 sec, 60°C for 30 sec, and final extension at 68°C for 12 min. The second PCR was performed with the first PCR products and attB adaptor primers (GGGGACAAGTTTGTACAAAAAAGCAGGCT, GGGGACCACTTTGTAACAGAAGCTGGGT). The PCR program was 98°C for 30 sec, 5 cycles of 94°C for 15 sec, 45°C for 30 sec, and 68°C for 12 min, 20 cycles of 94°C for 15 sec, 60°C for 30 sec, and final extension at 68°C for 12 min. The amplified attB-PCR segment was inserted into pDONR221 vector by BP Clonase (Thermo Fisher Scientific). Furthermore, the GVP 5’ UTR-Venus segment in the entry clone was recombined by LR Clonase (Thermo Fisher Scientific) into the destination vector, pMiDestF, which encodes Minos inverted repeats [(23), https://marinebio.nbrp.jp/ciona/]. Schematic depiction of the CiVP transgenic vector is shown in **Supplemental Figure 2A**.

Generation of Transgenic Ascidians

Transgenic ascidian was generated as described previously (24, 25). In brief, Minos transposase mRNA was synthesized using Megascript T3 kit, poly (A) tailing kit (Ambion, Carlsbad, CA) and Cap structure analog (New England Biolabs, Ipswich, MA). Both Minos mRNA and pMiDestF-CiVP 5’-UTR-Venus vector were electroporated into dechorionated and fertilized *Ciona* eggs (26). Subsequently, the electroporated eggs were cultured on agar-coated petri dishes with Millipore-filtered sea water. We raised the founder ascidian emitting Venus fluorescence, and its sperms were mated with wildtype ascidian eggs to obtain F1 progeny. Gametes of grown F1 progeny were then mated to generate transgenic F2 progeny.

RT-PCR Analysis of CiVP mRNA

Total RNA extracted from young adult ascidian ovaries was reverse-transcribed to the template cDNA using Superscript III (Thermo Fisher Scientific). RT-PCR was performed using GVP gene specific primers (TGCTCTAACATGGATTGG, GCACCTTTGTAAGAGAC), GAPDH gene specific primers (GACGAATTGGACGCT, GATGGTTGTGAAAACTCC), ExTaq polymerase (TAKARA BIO INC, Kyoto, Japan) and a thermal cycler (model GeneAmp PCR system 9700; Thermo Fisher Scientific). The PCR program was 94°C for 3 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and
72°C for 3 min. The PCR solutions were electrophoresed with 1.5% agarose gel.

**Generation of CiVP-Deleted Mutants by TALEN**

TALE Targeting sites of CiVP gene were determined using TALE Effector Nucleotide Targeter 2.0 (https://tale-nf.cac.cornell.edu/) (27). Left targeting site (TTTGACCGCTGTATTTT) contains sequences encoding the N-terminal Cys-Phe of CiVP (amino acid sequence: CFFRDGSDMDWYR), while Right targeting site (GCGGTACCAATTCCATGT) corresponds to the sequence encoding C-terminal Asn-Met-Asp-Trp-Tyr-Arg in CiVP. Plasmids encoding TALE binding to these CiVP sequences were constructed onto the TALEN backbone vector containing the ubiquitous promoter from Ciona elongation factor (EF1α) using Platinum Gate TALEN kit [Addgene, Cambridge, MA, USA; (28–30)]. NBRP web site (https://marinebio.nbrp.jp/ciona/) shows sequence of the backbone vector named as pBSGIEFla SanTAL-NG-2A-mcherry-ver181108. Schematic depiction of the CiVP TALEN construction verifying activity is shown in Supplemental Figure 2B. The EF1α-GiVP TALEN vectors were electroporated into Ciona embryos. At the tailbud stage, the electroporated animals having mCherry expression in the whole body were collected, and genomic DNA was extracted in bulk. The DNA region containing the TALEN target site was PCR amplified, and the PCR fragments were electrophoresed in 15% acrylamide gel. The PCR bands exhibiting mutation signatures were subcloned into a conventional vector, and their sequences were determined.

The TALEN repeats were digested with SalI and BamHI, and were subcloned into pTnI-TALEN-Ng:2A::mCherry with the In-fusion Cloning kit (TAKARA BIO INC). The GFP mutant line was generated by the electroporation-mediated germ cell regeneration method (31) using pTnl-GiVP-TALEN-L::2A::mCherry and pTnl-GiVP-TALEN-R::2A::mCherry. Schematic depiction of the GFP knockout line generation is shown in Supplemental Figure 2C. Genomic DNA was extracted from the sperm of Ciona adults using a Wizard genomic DNA purification kit (Promega). The GiVP region was amplified using ExTaq (TAKARA BIO INC), CiVP gene-specific primers (AGATACAGACTGTATAAGATTC, AACCTGAAATTGGCTCTGTTATCC), and a thermal cycler (GeneAmp PCR system 9700) by the PCR program: 94°C for 3 min, 50 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and final extension at 72°C for 7 min. The PCR products were sequenced after subcloning in TOPO cloning vector (Thermo Fisher Scientific) and/or analyzed by heteroduplex mobility shift assay (32) to screen GiVP heterozygous mutants. Sperm of the heterozygous mutants were mated with wildtype ascidian eggs to generate the F1 progeny. Moreover, CiVP homozygous mutants were generated using gametes of F1 ascidians. The homogeneity of GiVP-TALEN mutants was confirmed by electrophoresis of PCR products amplified using the aforementioned CiVP gene-specific primers and Bsa I-digests. Sequencing analysis demonstrated that the CGGGACTGCTCT sequence in the CiVP-coding region of the genomic DNA was substituted with CCGGTCTCT in the CiVP-TALEN mutants.

**Microscopic Observation of Ciona Ovaries**

Ciona ovaries were dissected from wild ascidians and mutant ascidians, and fixed in 4% paraformaldehyde/PBS at 4°C overnight. The fixed ovaries were dehydrated in ethanol and xylene, embedded in paraffin, cut into 10-μm thick sections, and were attached to Frontier coated glass slides (Matsunami Glass, Osaka, Japan). Paraffin was removed from the slides by xylene and ethanol. The deparaffinized sections were incubated in hematoxylin for 1 min, washed with water for 15 min, and incubated in eosin for 3 min. The stained sections were dehydrated in ethanol and xylene, and were observed under a microscope, ECLIPSE 600 (Nikon, Tokyo, Japan). The area of each ovary section was measured using Image J software and oocytes within the stained sections were counted to calculate oocyte occupancy.

**RNA-seq of Ciona Ovaries**

Total RNA was extracted from the ovaries of a CiVP homozygous mutant (CiVP mutant) and a wildtype ascidian using RNeasy mini kit (QIAGEN, Venlo, Netherlands). The quality of the RNA samples was evaluated using BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) with an RNA6000 Pico Chip. A 0.2-μg aliquot of total RNA from each sample was used to construct cDNA libraries using TruSeq Stranded mRNA Sample preparation kit (Illumina, San Diego, CA, USA), according to the manufacturer’s instructions. The resulting cDNA library was validated using BioAnalyzer with a DNA1000 Chip and quantified using Cycleave PCR Quantification Kit (TAKARA BIO INC). Single end sequencing over 101 cycles was performed using HiSeq1500 (Illumina) in the rapid mode. Total reads were extracted with CASAVA v1.8.2 (Illumina). Then, PCR duplicates, adaptor sequences, and low-quality reads were removed from the extracted reads as follows. Briefly, if the first 10 bases of the two reads were identical and the entire reads exhibited > 90% similarity, the reads were defined as PCR duplicates. Remaining reads were then aligned with Bowtie version 2.2.3 to the C. intestinalis genes (KH, ver.2013), which was downloaded from Ghost Database (http://ghost.zool.kyoto-u.ac.jp/download_kh.html). We defined fragments per kilobase of transcript per million mapped reads (FPKM) value > 5 as expression genes in the ascidian ovary. Furthermore, we defined genes satisfying the condition of "FPKM value of CiVP mutant ovary/FPKM value of wildtype ovary" > 2 as genes upregulated in the ovary of CiVP mutant. Likewise, genes satisfying the condition of "FPKM value of wildtype ovary/FPKM value of CiVP mutant ovary" > 2 were defined as upregulated in the ovary of wildtype ascidian.

**GO Analysis**

Putative genes for the transcripts were annotated based on a homology search of the Swissprot database under the condition of e-values < 0.0001, using Blast2GO software (version 4.1) with default parameters (33). Moreover, these ascidian genes were mapped and annotated using Blast2GO software with default parameters. The putative genes were annotated to Gene Ontology (GO) terms (biological process, molecular function, cellular component) using Blast2GO software.
and cellular component). Subsequently, the annotated biological information was compared using GO ‘is_a’ graphs as described previously (34, 35). The graphs were drawn with Cytoscape (http://www.cytoscape.org/). To quantify the enrichment of GO terms, we calculated enrichment scores as follows: where NX (GO) indicates the frequency of each GO term for GIVP mutant ovary (GIVP mutant << wildtype)- or wildtype ovary (wildtype << GIVP mutant)-specific genes (i.e., X), and Ntotal (X) indicates the frequency of Ci-VP mutant ovary (GIVP mutant << wildtype)- or wildtype ovary (wildtype << GIVP mutant)-specific genes (i.e., X) mapped to each GO term in the Blast2GO results. For collection, a pseudo-count was set as a fixed value.

Quantitative PCR
Total RNA was isolated from the ovary of wild ascidian and GIVP mutant. Each RNA was reverse-transcribed to the template cDNA at 50 C for 50 min. using the oligo (dT) anchor primer and Superscript III First Strand Synthesis Supermix (Thermo Fisher Scientific). Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Bio-rad, Hercules, California, USA) and CFX96 Real-Time System (Bio-Rad) with the ΔΔCt method as previously reported (35). In brief, Ct values for Ci-GAPDH gene expression were used as standard values, and ΔΔCt values were calculated for each gene according to the manufacturer’s instruction. Moreover, relative expression values were calculated from ΔΔCt values. Primer sequences are shown in Supplemental Table 1.

RESULTS AND DISCUSSION
Distribution of CiVPergic Neurons and Non-Neuronal Cells
To examine CiVP gene expression in detail, we constructed transgenic ascidians introduced by the 5-kb CiVP promoter region-conjugated with the ORF of a fluorescent protein, Venus. Fluorescence was detected in the cerebral ganglion of CiVP promoter-Venus-transgenic larvae (Figure 1A). This fluorescent signal was in good agreement with the localization of the GIVP mRNA in wildtype Ciona larva but not in any earlier developmental stages as observed in our previous study (36), suggesting that the fluorescence derived from CiVP promoter-Venus construct indicates the expression of the CiVP gene in the transgenic Ciona.

Observation of the transgenic ascidians showed that Venus-derived fluorescence disappeared during the body rotation period, and gradually increased during the protostigmata period. Venus-derived fluorescence was detected not only in the cerebral ganglion with the highest intensity but also in the oral siphon, atrial siphon, and ampulla in the transgenic juveniles (Figure 1B). In the transgenic adults, intense Venus fluorescence was detected in the cerebral ganglion (Figure 1C), consistent with the previous study demonstrating that the GIVP gene was expressed exclusively in neurons in the cerebral ganglion of wild-type adult ascidians (3). Moreover, fluorescence was detected in several nerves including, posterior nerve 1, posterior nerve 2, anterior lateral nerve 1, anterior lateral nerve 2, anterior medial nerve 1, and anterior medial nerve 2. Particularly, the posterior nerve 2 connects to visceral nerves (37), suggesting that CiVP regulates functions of the stomach and intestine. In addition, weak fluorescence was detected in the oral siphon and atrial siphon of adult ascidians (Figures 1D, E). The anterior lateral and anterior medial nerves project to the oral siphon, and posterior nerves project to the atrial siphon (37), suggesting that GIVP regulates functions in the siphons. Fluorescence was also detected in the ovary of the transgenic young adult ascidian (Figure 2A). Since fluorescence is observed in some cells in the ovary, CiVP is likely to act as an endogenous factor in the ovary. Moreover, fluorescent nerve fibers projected to several organs including the stomach, intestines, and ovary (Figure 2A). These results suggested that GIVP originating from the neural tissue is involved in regulation of functions of the digestive tracts and ovary in the young adult ascidian. In our previous study, RT-PCR indicated that the CiVP gene was not expressed in the ovary of adult Ciona (3). To confirm the GIVP gene expression in the ovary of young adult Ciona, we performed RT-PCR for GIVP mRNA using three different wildtype young adult ascidian ovaries. RT-PCR for GIVP mRNA proved that the CiVP gene was expressed specifically in the ovaries of young adult ascidians (Figure 2B), although the elucidation of the biological significance of the gene expression awaits further study.

CiVP MUTANTS
To investigate biological functions of CiVP in vivo, we generated mutants of CiVP gene using the TALEN method. As a deletion site of the CiVP gene, we targeted the genome sequence encoding the N-terminal region of CiVP, since VP/OT family peptides including CiVP conserve Cys1 and Cys6 that form a disulfide bridge, and are responsible for activities of the family peptides (3, 7, 21). Thus, deletion of either of the cysteines is expected to lead to a loss of CiVP functions.

We selected the heterozygous mutants in which the CiVP gene has lost four nucleotides corresponding to Asp5 and Cys6 (Figure 3). CiVP mutant sperms were mated with wildtype ascidian eggs to generate F1 progeny containing CiVP heterozygous mutation. F2 progeny harboring CiVP homozygous mutants were generated using gametes of the CiVP heterozygous mutants. The homozygous mutants were designated CiVP mutants. At the larval stage, CiVP homozygous mutants are indistinguishable from wildtype siblings, and the mutants metamorphosed to juveniles with normal morphology. Likewise, CiVP mutants exhibited growth similar to wildtype ascidians until reaching 2-cm body length. In contrast, growth of CiVP mutants gradually delayed compared with that of wildtype ascidians after 2-cm body length, and the growth of CiVP mutants stopped earlier than that of wildtype ascidians. As shown in Figure 4, the average body length of CiVP mutants and wildtype ascidians are approximately 3.5 cm and 6.2 cm, respectively. These results suggest that CiVP is involved in growth after ascidians reach a length of 2 cm. This is the first demonstration of a negative effect of deletion of an OT/VP gene deletion on total body growth of an animal.
Previously, the cognate CiVP receptor gene was shown to be expressed in the ovary (3). Furthermore, we substantiated that CiVP regulates oocyte maturation and ovulation (21). To examine the effect of CiVP on the whole ovary, we observed the ovaries of CiVP mutant and wildtype Ciona at the reproductive stage. Ciona follicles are classified into four growth stages (stage I – IV) (38). Pre-vitellogenic follicles, vitellogenic follicles, and post-vitellogenic follicles are classified into stages I, II, and III, respectively. Size of stage I, II, and III follicles are approximately 50, 70, and 100 μm in diameter, respectively. Stage I - III follicles are infertile, while stage IV follicles are fertile and ovulated (21, 38). We counted all hematoxylin/eosin-stained follicles in ovarian sections of CiVP mutant and wildtype ascidians. A total of 476 follicles were present in the section of CiVP mutant ovary, while 354 follicles were present in the section of wildtype Ciona ovary. In contrast, only 7 post-vitellogenic (stage III) follicles were detected in the section of CiVP mutant ovary, although 35 stage III follicles were observed in the section of wildtype Ciona ovary. In addition, we calculated the number of stage III follicles in a given area (mm²) of ovarian section of CiVP mutant and wildtype ascidians, revealing that the occupancy of stage III follicles in the mutant ovary section was 1.63 oocytes/mm², which was markedly lower than 6.34 follicles/mm² in the wildtype ovary section (Figure 5 and Table 1). Moreover, stage III follicles constituted 1.4% of all follicles in the
CiVP mutant ovary, and 9.9% of all follicles in the section of the wildtype ascidian ovary (Figure 5 and Table 1). Since pre-vitellogenic follicles (stage I) and vitellogenic follicles (stage II) are more abundant in the CiVP mutant ovary, the number of all follicles per mm² in the mutant section was approximately 1.7-fold greater than that of all follicles per mm² in the wildtype ovarian section. Oocyte maturation is induced by CiVP in stage III follicles, leading to ovulation of fertile follicles (stage IV) (21, 38). Combined with these findings, the present results are consistent with the biological roles of CiVP, and also suggests that CiVP is involved in the stage I to III follicle growth. In other words, in the CiVP mutants, suppression of growth of stage II to -III may lead to the accumulation of stage I and stage II follicles, given that they are much smaller than stage III follicles, and thus a greater number of stage I and II follicles can occupy the ovary, compared with stage III follicles. This view is supported by the present results demonstrating that the CiVP mutant ovary harbors more follicles but fewer stage III follicles than the wildtype ovary (Figure 5 and Table 1).

OT/VP gene- or their receptor gene-knockout mice exhibited no obvious deficiencies in fertility, follicle maturation, or reproductive behavior, although OT gene-knockout mice
cannot release milk and OT receptor gene-knockout mice fail to lactate and exhibited a decrease in maternal behaviors [(39, 40), and Mouse Genome Informatics; http://www.informatics.jax.org]. These findings confirm that follicles normally grow in VP/OT signaling-deficient mutants. Likewise, analyses of nematodes, C. elegans indicated that the null mutants of the OT/VP superfamily peptide gene are as viable and fertile as wildtype nematodes, and exhibited normal egg-laying behavior and number of progeny but male nematodes exhibited low success in mating behavior (14). In addition, injection of the cognate OT/VP peptides resulted in induction of egg-laying behavior of annelids (2, 7, 18, 19). In the present study, stage III follicles were reduced in the ovary of CVP mutants (Figure 5 and Table 1), demonstrating that CiVP is responsible for follicle growth in addition to oocyte maturation and ovulation (21). In combination, the present results suggest species-specific biological roles of OT/VP superfamily peptides in reproductive functions such as follicle growth. Investigation of the underlying molecular mechanisms is underway.

GO Analysis of the Ovary of CiVP Mutants

RNA-seq for ovaries of the CiVP mutant and wildtype ascidian using Hiseq 1500 yielded 40 million and 44 million reads,
respectively for 101 paired-end reads. The resultant fastq files were deposited in SRA database (accession no. SRR12791386 and SRR12791387). Reads originating from each ovary were mapped against the C. intestinalis gene database (http://ghost.zool.kyoto-u.ac.jp/cgi-bin/gb2/gbrowse/kh/) to a total of 53,083 and 52,637 genes, respectively. The gene expression levels were assessed based on FPKM values were used. A total of 18,377 and 21,933 non-overlapping genes exhibiting FPKM values >5 were eventually identified in the CiVP mutant and wildtype ovaries, respectively. Figure 6 shows a scatter plot of FPKM values of these genes.

GO analysis using Blast 2 GO indicated that frequent GO terms were similar between the CiVP mutant and wildtype ovaries. Subsequently, we calculated the ratio of the FPKM values for CiVP mutants to the FPKM values for wildtype ascidians to detect genes upregulated in the premature or mature ovaries. Subsequently, we calculated the ratio of the FPKM values for CiVP mutants to the FPKM values for wildtype ascidians to detect genes upregulated in the premature or mature ovaries, indicating that 1,976 genes and 5,644 genes were upregulated in the CiVP mutant and wildtype ovaries, respectively. Figure 6 shows a scatter plot of FPKM values of these genes.

TABLE 1 | The number and ratio of stage III follicles in a section of the ovaries of wildtype Ciona and CiVP mutant (n=2).

| Area of tissue slice (mm²) | Number of stage III follicles | Number of stage III follicles per mm² (follicles/mm²) | Number of all follicles | Number of all follicles per mm² (oocytes/mm²) | Proportion of stage III follicle among all follicles (%) |
|---------------------------|-------------------------------|-----------------------------------------------------|------------------------|-----------------------------------------------|-----------------------------------------------------|
| wildtype ascidian          | 5.52                          | 35                                                  | 6.34                   | 354                                           | 64.1                                                | 9.9                                                 |
| CiVP mutant               | 4.30                          | 7                                                   | 1.63                   | 476                                           | 110.7                                               | 1.4                                                 |

of e-value <0.0001 indicated that 86.2% (n = 1,704) and 96.8% (n = 5,463) of genes upregulated in the ovary of CiVP mutants and wildtype ascidians, respectively, were homologous to known proteins. Furthermore, GO data enrichment scores for these genes were calculated to examine the features of the ovary of the CiVP mutants. Annotated biological information was compared between CiVP mutant and wildtype ovaries using a heat map in graph view (Figure 7). Red indicates high enrichment score for the CiVP mutant, and blue shows low enrichment score. For simple interpretation, a GO term with a GO level <2 or a GO term with an enrichment score between −1.2 and 1.2 would be represented as a zero-sized node. Figure 7 and Table 2 show data for three enrichment analyses for the representative GO categories biological process, cellular component, and molecular function. Enrichment analysis of biological process indicates that genes related to biosynthetic processes were upregulated in the ovary of CiVP mutants (Figure 7A and Table 2). Furthermore, genes related to ribonucleoprotein were also upregulated in the mutant ovary (Figure 7A and Table 2). On the other hand, genes related to transport and localization were downregulated (Figure 7A and Table 2). Enrichment analyses of cell components showed that genes related to ribosome were upregulated in the CiVP mutant ovary (Figure 7B and Table 2). In contrast, enrichment analyses of cell components indicated that genes related to various organelles were downregulated in the ovary of a CiVP mutant (Figure 7B and Table 2). Expression profiles of genes related to the biological process and cellular component categories suggested that formation of organelles in ovary cells is suppressed by deletion of CiVP, whereas the synthesis of biomolecules appears to be activated (Table 2), ultimately disrupting cellular homeostasis or normal cell division. Enrichment analysis of the molecular function category indicated that genes related to binding and phosphorylation were downregulated in the ovary of CiVP mutants, whereas genes related to the nucleus or ribosomes were upregulated (Figure 7C and Table 2). The expression of genes related to nucleotide and protein synthesis was upregulated in the ovary of CiVP mutants, whereas the expression of genes related to organelles was downregulated (Figure 7C and Table 2). Thus, formation and activation of organelles are likely to be suppressed in the ovary of CiVP mutants, although nucleotides and proteins are synthesized. In Ciona ovarian follicles, accessory cells surrounding the oocytes, such as test cells, are produced from stage I to II, and are actively proliferated from stage II to III (38). Particularly, the number of accessory cells markedly

FIGURE 6 | Comparative scatter plot analysis of gene expression levels in the ovary of CiVP mutant and wildtype Ciona. Horizontal axis shows FPKM values of the CiVP mutant, whereas the vertical axis shows FPKM values for wildtype Ciona.
FIGURE 7 | Transcriptomic analyses of CiVP mutant and wildtype Ciona ovaries. GO enrichment analysis of 343 differentially expressed genes in premature and mature ovaries. GO terms were categorized as 344 "(A), Biological Process" (BP), "(B), Molecular Function" (MF), and "(C), Cell Component" (CC). Each node represents a GO term. Branches of GO hierarchical trees not exhibiting significantly enriched GO terms are not shown. The size of each node circle indicates the number of genes with that GO term. The color of each node indicates the enrichment score. Red and blue indicate high enrichment scores of genes expressed in the ovary of CiVP mutant and wildtype ascidians, respectively. Edges indicate ‘is_a’ connections between GO terms.
increases during follicle growth from stage II to III (38). Consequently, it is presumed that formation and activation of organelles are upregulated according to the proliferation of accessory cells in the ovary of wildtype ascidians, whereas genes related to organelles are downregulated in the ovary of CiVP mutants. These findings are compatible with the upregulated expression of genes related to nuclei and ribosome in the ovary of CiVP mutant (Figure 7 and Table 2).

Quantitative PCR for genes detected by RNA-seq and GO analysis between wildtype and CiVP mutants revealed 1.5 to 2-fold differential expression of various genes. For example, ceramide synthase homologous gene showed 1.5-fold lower expression in the ovary of CiVP mutant than that of wildtype ascidian (Figure 8). Ceramide is known to participate in the removal of low-quality early follicles as a lipid mediator in mammals (41, 42). As shown in Figure 5, the ovary of CiVP mutant possesses far fewer stage III (post-vitellogenic) follicles than that of wildtype, demonstrating that normal growth from stage II to III is inhibited by occupation of early follicles that cannot grow. Altogether, the present study suggests that CiVP is involved in quality control of follicle maturation via upregulation of ceramide synthase gene expression.

Expression of the PKCβ homologous gene was downregulated 1.6-fold in the ovary of CiVP mutants versus wildtype ascidians (Figure 8). PKCβ has multi-functions including construction of organelle in mammals. For example, PKCβ-knockout rat mast cells exhibited reduced vesicle fusion (43), indicating that PKCβ is an important factor for vesicle transport during the secretion of signal molecules as well as

**TABLE 2 | Representative GO ID regulated in CiVP mutant.**

| category | ID               | regulation in CiVP mutant |
|----------|------------------|---------------------------|
| BP       | GO:0006139       | nucleobase-containing compound metabolic process up |
| BP       | GO:0009059       | macromolecule biosynthetic process up |
| BP       | GO:0034645       | cellular macromolecule biosynthetic process up |
| BP       | GO:0019439       | aromatic compound catalytic process up |
| BP       | GO:004270        | cellular nitrogen compound catalytic process up |
| BP       | GO:0022613       | ribonucleoprotein complex biogenesis up |
| BP       | GO:0071826       | ribonucleoprotein complex subunit organization up |
| BP       | GO:0016192       | vesicle-mediated transport down |
| BP       | GO:0048193       | Golgi vesicle transport down |
| BP       | GO:0046907       | intracellular transport down |
| BP       | GO:0015031       | protein transport down |
| BP       | GO:00016197      | endosomal sorting complex required for transport down |
| BP       | GO:0051049       | regulation of transport down |
| BP       | GO:0060627       | regulation of vesicle-mediated transport down |
| BP       | GO:006341        | regulation of cellular localization down |
| BP       | GO:0032880       | regulation of protein localization down |
| CC       | GO:0005840       | ribosome up |
| CC       | GO:0044391       | ribosomal subunit up |
| CC       | GO:0030684       | preribosome up |
| CC       | GO:000532        | small nuclear ribonucleoprotein complex up |
| CC       | GO:0005681       | spliceosomal complex up |
| CC       | GO:0005783       | endoplasmic reticulum down |
| CC       | GO:004431        | Golgi apparatus part down |
| CC       | GO:0000139       | Golgi membrane down |
| CC       | GO:0005794       | Golgi apparatus down |
| CC       | GO:004440        | endosomal part down |
| CC       | GO:0010008       | endosome membrane down |
| CC       | GO:0031410       | cytoplasmic vesicle down |
| CC       | GO:0044433       | cytoplasmic vesicle part down |
| CC       | GO:0030659       | cytoplasmic vesicle membrane down |
| CC       | GO:0016023       | cytoplasmic membrane-bounded vesicle down |
| MF       | GO:0003735       | structural constituent of ribosome up |
| MF       | GO:0003723       | exportin-5 recognized 3’ overhang of pre-miRNA up |
| MF       | GO:004518        | nuclease activity up |
| MF       | GO:004652        | enzyme binding down |
| MF       | GO:005062        | coenzyme binding down |
| MF       | GO:0032403       | protein complex binding down |
| MF       | GO:0051082       | unfolded protein binding down |
| MF       | GO:0008134       | transcription factor binding down |
| MF       | GO:0044389       | ubiquitin-like protein ligase binding down |
| MF       | GO:0016773       | phosphoryltransferase activity down |
| MF       | GO:0016301       | phosphorylation of Cx43 by c-src down |
organelle formation. This function of PKCβ coincides with the result of GO analysis demonstrating that genes related to organelles were downregulated in the ovary of CiVP mutants (Figure 7 and Table 2). PKCβ thus appears to be related to impaired follicle growth in Ciona at least in part via a disruption of vesicle transport, although the investigation of the detailed molecular mechanisms awaits further study.

Expression of the MLH3 gene was upregulated 2.1-fold in the ovary of CiVP mutants versus wildtype ascidians (Figure 8). MLH3 is known to repair DNA mismatch during DNA replication and meiosis (44). It is noteworthy that ascidian meiotic competence is acquired in stage III follicles (45) which are far fewer in the ovary of CiVP mutants (Figure 5). In combination, these results suggest that MLH3 plays a major role in DNA replication in the Ciona ovary. Furthermore, as stated above, Ciona follicle accessory cells—such as test cells—are actively proliferated at stage I and II (38). Consequently, the present study, combined with these findings, supports the view that higher expression of MLH3 is functionally implicated with more abundant immature follicles (stage I and II) in the ovary of CiVP mutants, compared with those of the wildtype. Taken together, these gene expression profiles suggest that CiVP plays a variety of roles in the control of the growth of early-stage follicles via regulation of diverse genes, although verification of the precise molecular mechanisms awaits further study.

In conclusion, we have presented the CiVPergic innervation and phenotypes and gene expression profiles of CiVP mutant Ciona. This is the first demonstration of both neuropeptide promoter-transgenic and neuropeptide gene-deleted Ciona adults, in particular, insufficient growth of the whole body and ovarian follicles in Ciona adults. Consequently, the present study will pave the way for investigations of not only various biological roles of CiVP but also the functional evolution of VP/OT family peptides.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

TK and HS conceived and designed the study. AH, TH, and YS constructed the transgenic and gene-deletion ascidians. AS, SM, and TK conducted RNA-seq experiments and analyzed the data. TK performed quantitative PCR analyses. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.668564/full#supplementary-material
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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