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Molecular Cloning of *AZIN2* and its Expression Profiling in Goose Tissues and Follicles

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Original article

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Ornithine decarboxylase antizyme inhibitor 2 (AZIN2) plays key roles in regulating the biosynthesis and content of polyamines and other amines such as serotonin and histamine. The *AZIN2* coding sequence in the goose was cloned and analysed in this experiment. *AZIN2* expression levels in goose tissues and follicles were also measured. A full-length *AZIN2* coding sequence (GenBank accession no. MF939648) encoding a 457-amino acid protein was 1374 bp in length. The molecular weight of putative AZIN2 protein was 49.10 kDa. The mRNA expression of *AZIN2* was not observed in heart, adrenal gland, breast muscle, thigh muscle and pineal gland tissues in the goose. The highest and lowest expression levels of *AZIN2* in all examined tissues were observed in cerebrum and kidney tissues, respectively. The mRNA expression levels of *AZIN2* in the cerebrum, cerebellum and hypothalamus were 61.95-, 15.87- and 15.04-fold higher compared to the liver, respectively. The mRNA expression of *AZIN2* was observed in all examined follicles. The mRNA expression level of *AZIN2* was 6.48-fold higher in the F1 follicle compared to the SWF. Taken together, *AZIN2* mRNA has a restricted pattern of expression in the goose. AZIN2 may play important roles in modulating physiological functions of the brain and regulating follicular development and ovulation of goose ovaries.

Key words: Goose, ornithine decarboxylase, ornithine decarboxylase antizyme inhibitor 2, follicle.

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Polyamines regulate cell proliferation, differentiation, apoptosis, reproduction, and cancer (THOMAS & THOMAS 2001; LEFEVRE *et al*. 2011). Polyamine levels are precisely regulated through various processes including polyamine biosynthesis, catabolism and transport (PEGG 2009). An autoregulatory circuit consisting of ornithine decarboxylase (ODC), ornithine decarboxylase antizyme (OAZ) and ornithine decarboxylase antizyme inhibitor (AZIN) control the intracellular level of polyamines (HOSHINO *et al*. 2005; OLSEN & ZETTER 2011; QIU *et al*. 2017). OAZs inhibit the activity of ODC and target its degradation. AZIN binds to OAZ with a higher affinity than ODC and rescues ODC from the ODC-OAZ complex (LIU *et al*. 2011; QIU *et al*. 2017).

Up to date, the AZIN family was made up of AZIN1 and AZIN2 proteins (KAHANA 2009; MA *et al*. 2015). Both AZIN1 and AZIN2 bind to OAZ with the same affinity. AZINs are highly homologous to ODC but lack decarboxylase activity (MURAKAMI *et al*. 1996). Recently, several studies demonstrated that AZIN2 enhanced the stability and activity of ODC and polyamine influx through counteracting the inhibition of OAZ (LOPEZ- CONTRERAS *et al*. 2006; KANERVA *et al*. 2008).

AZIN2 has a significant role in maintaining polyamine homeostasis and regulating cell proliferation, similar to that found for AZIN1 (KEREN-PAZ *et al*. 2006; SILVA *et al*. 2015). High expression of AZIN2 is found in the testis and brain and differentiated resting cells (PITKANEN *et al*. 2001; MAKITIE *et al*. 2010; LOPEZ-GARCIA *et al*. 2013). The expression level of AZIN2 is positively correlated to the polyamine level in the brain of Alzheimer's patients (MAKITIE *et al*. 2010; INOUE *et al*. 2013). Recently, several studies have suggested that AZIN2 plays a role in regulating the biosynthesis of serotonin and histamine and may have a role in the endocrine function of adrenal glands and pancreas (KANERVA *et al*. 2009; LOPEZ-GARCIA *et al*. 2013; ACOSTA-ANDRADE *et al*. 2016). These reports suggest that AZIN2 has multiple biological functions. Our previous study cloned and characterized the *AZIN1* gene of the Sichuan white goose, and implied that AZIN1 played important roles in follicular development (MA *et al*. 2015). Thus far, the goose *AZIN2* gene and its expression profiles remain to be determined. To characterize the goose *AZIN2* gene, we cloned the *AZIN2* cDNA sequence and measured mRNA expression levels of *AZIN2* in different tissues and ovarian follicles.

Materials and Methods

Preparation of experimental animals and tissue collection

All experimental procedures were performed in accordance with the Institutional Review Board (IRB14044) and the Institutional Animal Care and Use Committee of the Sichuan Agricultural University under permit number DKY-B20140302. The heart, liver, spleen, lungs, kidneys, adrenal glands, breast muscles, thigh muscles, cerebrum, cerebellum, pineal gland, hypothalamus, pituitary gland, uterus, follicles and ovarian stroma in laying Sichuan white geese were collected and prepared on ice as described previously (MA *et al*. 2015).

Total RNA extraction and amplification of *AZIN2*

Total RNA from goose tissue samples was extracted with a Trizol reagent (Takara, Dalian) following the manufacturer's instructions. The reverse transcription of each RNA sample isolated from all examined tissues was completed using a PrimeScript® RT reagent Kit (Takara). Primers for amplifying the *AZIN2* coding sequence were as follows:
AZIN2-1: 5'-CACGAGTGCCGTCACACTTT-3', 5'-TATCACAGCAGCGATCTCCTC-3';
AZIN2-2: 5'-GCCAACAACCTCCACAGCCT-3', 5'-ACGACGCGGCGATGGTGTAT-3' and
AZIN2-3: 5'-AGAAGCCTTGCCCAGACCA-3', 5'-AAGAGGCCCGCACCGATCG-3'. The 50 μl reaction was composed of 2 μl of cDNA, 0.5 μl of each primer (10 μmol/l), 33.5 μl of sterile Milli-Q water, 8 μl of dNTP, 5 μl of LA PCR™ Buffer II and 0.5 μl of TaKaRa LA Taq HS (Takara Bio Inc.). The PCR was performed by the program: 95°C for 5 min, 60°C for 40 s, 72°C for 40 s, 35 cycles. *AZIN2* gene fragments were cloned and sequenced following standard procedures as described previously (KANG *et al*. 2014).

Bioinformatic analysis

The ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was employed to analyse the coding sequence of goose *AZIN2*. Online NCBI Blast was used to align nucleotide and amino acid sequences of *AZIN2* homologs. The physiochemical properties and subcellular distribution were analysed using the Protamina (http://web.expasy.org/protparam/) and PSORT II (http://www.genscript.com/psort.html), respectively. The secondary structure of the putative AZIN2 protein was analysed by SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html). Based on the neighbor-joining method with 1000 bootstrap replicates, a phylogenetic tree was also constructed by the MEGA program.

qRT-PCR

Expression levels of *AZIN2* were measured in an iCycler CFX96 using iTaqTM SYBR® Green Supermix (Bio-Rad, USA). Primers for amplifying *AZIN2* and *GAPDH* were as follows:
AZIN2-S: 5'-CGCTGCTGTGATAAACTCTG-3', 5'-CTTCCTTGGCGGTGATGC-3' and
GAPDH: 5'-GTGGTGCAAGAGGCATTGCTGAC-3', 5'-GCTGATGCTCCCATGTTCGTGAT-3'.
Briefly, the 20 μl reaction consisted of 1 μl of cDNA, 10 μl of Supermix, 0.4 μl of 10 μmol/l of each primer, and 8.2 μl of ddH_2O. PCR conditions for *AZIN2* was 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, 62°C for 30 s and 72°C for 30 s, and then an 80 cycles melting curve was performed. The relative mRNA expression levels of *AZIN2* with three replicates for each sample were calculated relative to *GAPDH* using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance using the SAS 9.2 statistical software for Windows (SAS Institute Inc., NC, USA) followed by Duncan's multiple range test. All values are presented as the mean ±SEM. A $P<0.05$ was considered to be statistically significant.

Results

Characteristics of goose *AZIN2*

Three partial fragments of the *AZIN2* gene were amplified and sequenced. The *AZIN2* cDNA assembled from the three fragments was 1398 bp in length including a 1374 bp coding sequence that encoded a 457-amino acid protein. The *AZIN2* nucleotide sequence was deposited in the GenBank (GenBank accession no. MF939648). The *AZIN2* cDNA sequence of the goose (*Anser cygnoides*) shared 88%, 69%, and 69% sequence identity with the *AZIN2* genes of *Gallus gallus* (NM_001293656.1), *Mus musculus* (NM_001301841.1), and *Homo sapiens* (NM_001293562.1), respectively. The putative AZIN2 amino acid sequence shared 86%, 59%, and 58% identity to *Gallus gallus* (NP_001280585.1), *Mus musculus* (NP_766463.1), and *Homo sapiens* (NP_001280491.1), respectively.

AZIN2 protein sequence analysis

The theoretical pI of the putative 49.10 kDa AZIN2 protein was 5.28 in the goose. Putative AZIN2 instability index was computed as 48.57. The grand average of hydropathicity was 0.026. AZIN2 secondary structure was predicted to consist of 39.82% alpha helix, 18.60% extended strand, 7.44% beta turn and 34.14% random coil. The predicted subcellular location of goose AZIN2 protein was 47.8% cytoplasmic, 26.1% nuclear, 17.4% mitochondrial, 4.3% cytoskeletal, and 4.3% plasma membrane. The putative goose AZIN2 protein contained a 238-amino acid pyridoxal-dependent decarboxylase (PDX) pyridoxal binding domain, a 123-amino acid PDX C-terminal sheet domain, a 19-amino acid Orn/DAP/Arg decarboxylases family 2 pyridoxal-P attachment site and an 18-amino acid Orn/DAP/Arg decarboxylase family 2 signature 2 (Fig. 1). Based on the putative AZIN2 amino acid sequence, a phylogenetic

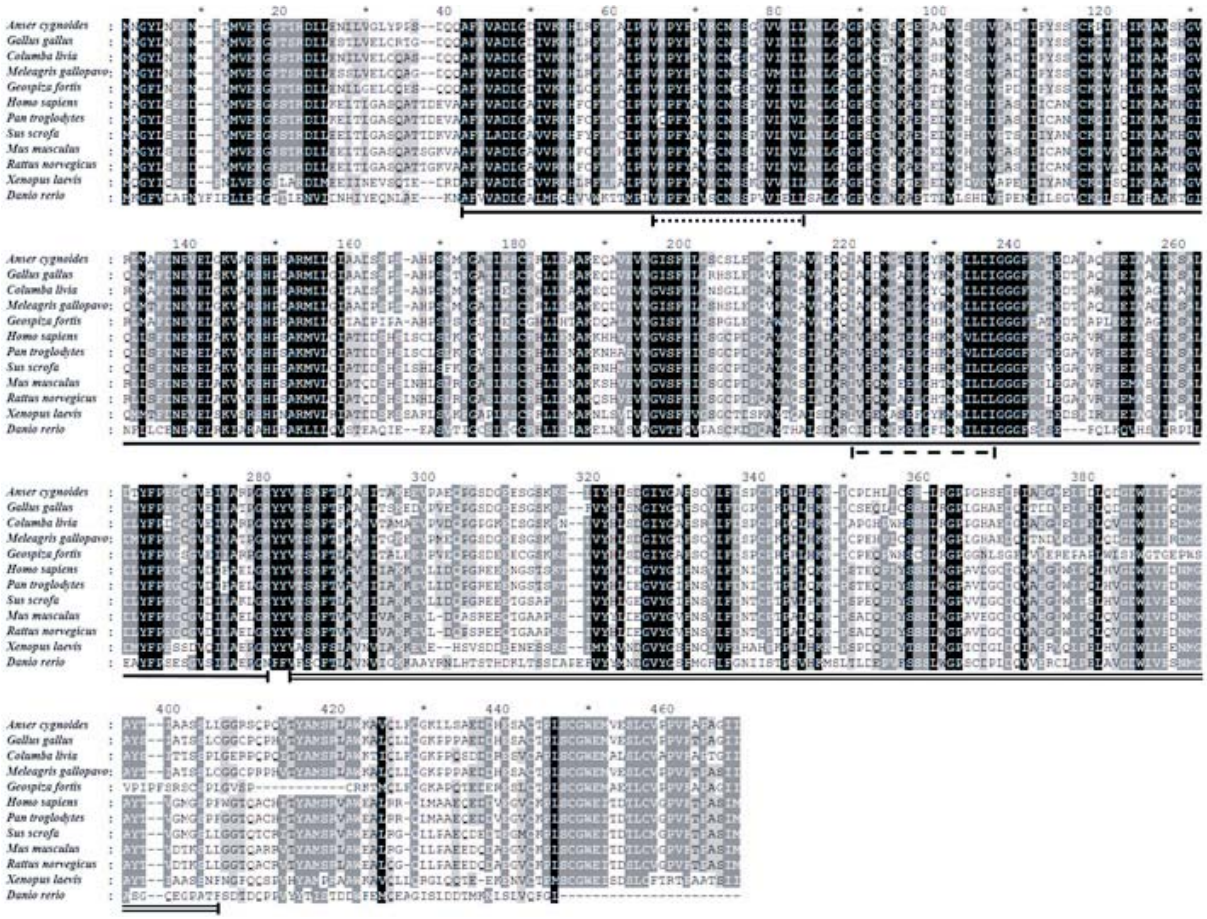

Fig. 1. Multiple alignments of putative AZIN2 amino acid sequences from goose and other species. The single line denotes a pyridoxal-dependent decarboxylase pyridoxal binding domain (position: A43-R280); the double lines denote a pyridoxal-dependent decarboxylase C-terminal sheet domain (position: V283-L405); the dotted line denotes an Orn/DAP/Arg decarboxylases family 2 pyridoxal-P attachment site (position: V65-L83); and the dashed line denotes an Orn/DAP/Arg decarboxylases family 2 signature 2 (position: I220-I237). Accession numbers for sequences used in the alignment: *Anser cygnoides*, *Gallus gallus* (NP_001280585.1), *Columba livia* (NP_001280682.1), *Meleagris gallopavo* (NP_001281204.2), *Geospiza fortis* (NP_001280680.1), *Homo sapiens* (NP_001280491.1), *Pan troglodytes* (XP_016814080.1), *Sus scrofa* (NP_001116665.1), *Mus musculus* (NP_766463.1), *Rattus norvegicus* (NP_001014283.2), *Xenopus laevis* (NP_001079692.1), *Danio rerio* (NP_001007160.2).

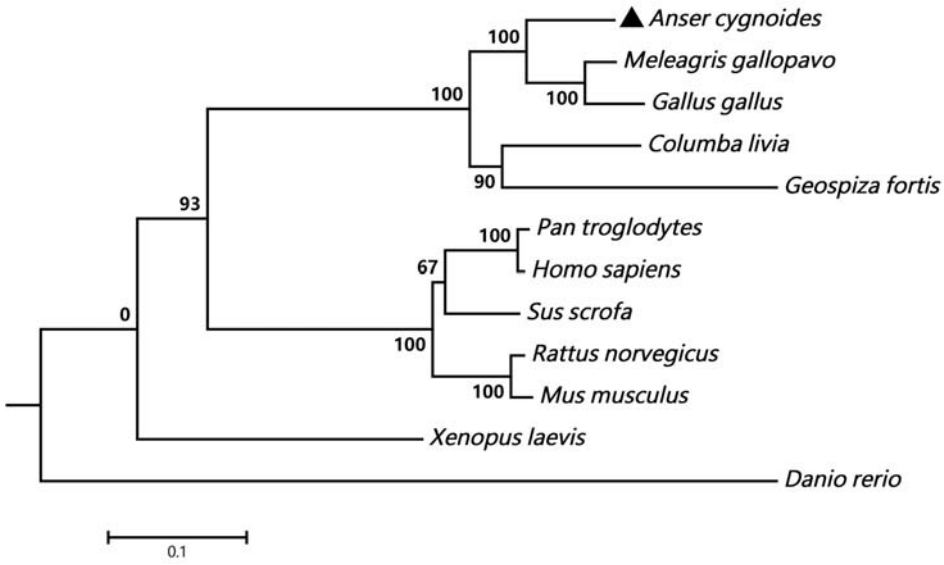

Fig. 2. Phylogenetic tree of AZIN2 amino acid sequences. The AZIN2 sequences shown were as follows: *Anser cygnoides*, *Gallus gallus* (NP_001280585.1), *Columba livia* (NP_001280682.1), *Meleagris gallopavo* (NP_001281204.2), *Geospiza fortis* (NP_001280680.1), *Homo sapiens* (NP_001280491.1), *Pan troglodytes* (XP_016814080.1), *Sus scrofa* (NP_001116665.1), *Mus musculus* (NP_766463.1), *Rattus norvegicus* (NP_001014283.2), *Xenopus laevis* (NP_001079692.1), *Danio rerio* (NP_001007160.2).

tree was constructed, and goose AZIN2 was most similar to that found in *Meleagris gallopavo* and *Gallus gallus* (Fig. 2).

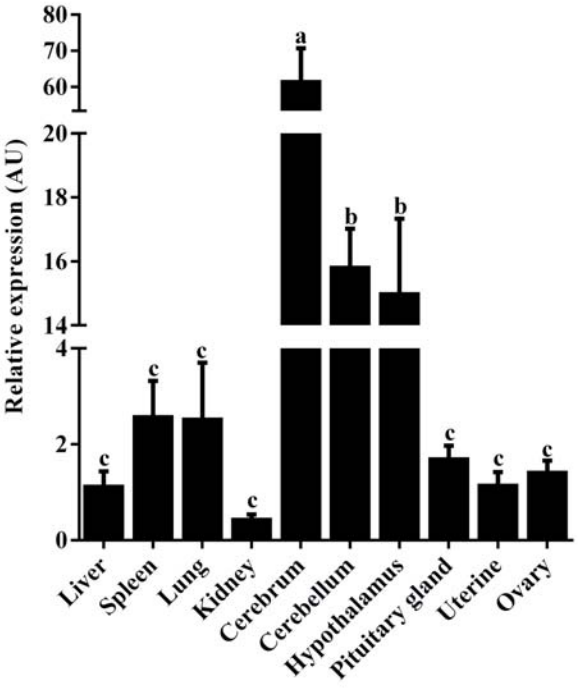

Fig. 3. Expression levels of the *AZIN2* gene in goose tissues. Expression levels of the *AZIN2* gene were normalized to *GAPDH*. Expression levels calculated by the $2^{-\Delta\Delta Ct}$ method were presented in arbitrary units (AU). Values are expressed as the mean ±SEM. Bars without a common letter are significantly different (P<0.05).

AZIN2 expression profiling in goose tissues

The mRNA expression of *AZIN2* was not observed in heart, adrenal gland, breast muscle, thigh muscle and pineal gland tissues in the goose using qRT-PCR. These results were also confirmed by semi-quantitative reverse transcription PCR (data not shown). The highest and lowest expression levels of *AZIN2* in all examined tissues were observed in cerebrum and kidney tissues, respectively (Fig. 3). The mRNA expression levels of *AZIN2* were significantly higher in cerebrum, cerebellum and hypothalamus tissues than in other examined tissues (P<0.05). *AZIN2* expression levels in the cerebrum, cerebellum and hypothalamus were 61.95-, 15.87- and 15.04-fold higher compared to the liver (P<0.05), respectively. We did not find significant differences in expression levels of *AZIN2* in the liver, spleen, lungs, kidneys, pituitary gland, uterus and ovary in the goose (P>0.05).

AZIN2 expression profiles in goose ovarian follicles

The mRNA expression of *AZIN2* was observed in all examined follicles (Fig. 4). During follicular development, significant difference in the mRNA expression levels of *AZIN2* was not observed among the SWF, SYF and F5-F2 follicles (P>0.05). The mRNA expression level of *AZIN2* in the F1 follicle was the highest in all examined follicles and was 6.48-fold higher compared to in the SWF (P<0.05). Significant difference in *AZIN2* expression levels were not detected among any POF follicles (P>0.05).

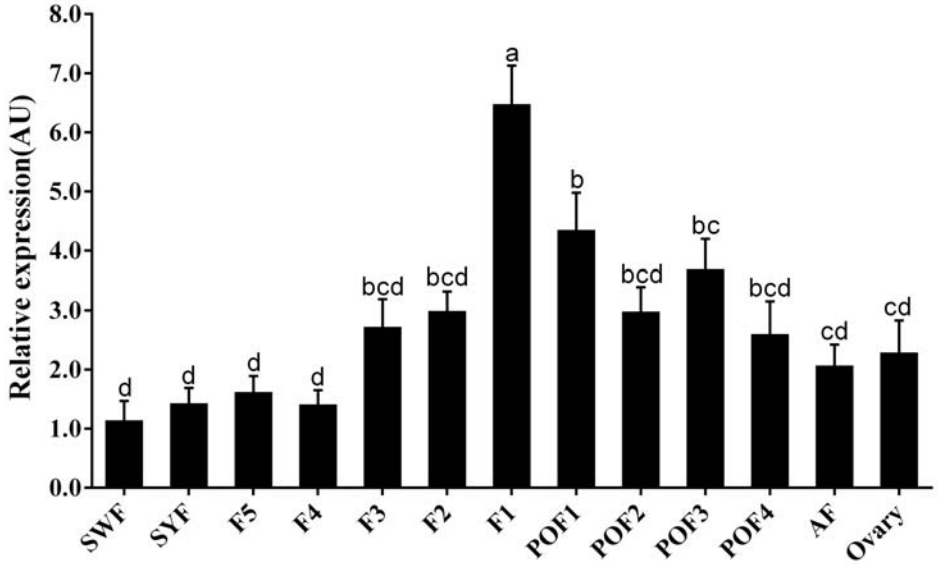

Fig. 4. Expression levels of the *AZIN2* gene in goose follicles and ovary. Expression levels of the *AZIN2* gene were normalized to *GAPDH*. Expression levels calculated by the $2^{-\Delta\Delta Ct}$ method were presented in arbitrary units (AU). Values are expressed as the mean ±SEM. Bars without a common letter are significantly different ($P<0.05$).

Discussion

The goose *AZIN2* coding sequence was cloned and characterized for the first time in this study. The putative AZIN2 protein in the goose had a molecular mass of 49.10 kDa, similar to that of mammal AZIN2 (50 kDa) and goose AZIN1 (50 kDa) (LOPEZ-CONTRERAS *et al.* 2010; MA et al. 2015). As in the case of AZIN1, goose AZIN2 was also a labile protein (PITKANEN *et al.* 2001; RAMOS-MOLINA *et al.* 2014; MA *et al.* 2015). Human AZIN2 retains 45% identity and 66% similarity to AZIN1 at the amino acid level (PITKANEN *et al.* 2001; OLSEN & ZETTER 2011). In this study, our data showed that the identity and the similarity between goose AZIN2 and AZIN1 were 41 and 64%, respectively. The theoretical pI of the putative AZIN1, AZIN2 and ODC protein of the goose was 4.79, 5.28 and 5.00, respectively (MA *et al.* 2015). In the goose, the putative ODC secondary structure consisted of 35.87% alpha helix, 23.7% extended strand, 8.91% beta turn and 31.52% random coil (our unpublished data). MA *et al.* (2015) reported that goose AZIN1 secondary structure was predicted to consist of 36.00% alpha helix, 16.89% extended strand and 47.11% random coil (MA *et al.* 2015). In this study, goose AZIN2 secondary structure consisted of 39.82% alpha helix, 18.60% extended strand, 7.44% beta turn and 34.14% random coil. In geese, the subcellular location of putative AZIN1, AZIN2 and ODC proteins was different. The subcellular distribution of the AZIN1 protein was predicted to be 73.9% in cytoplasmic, 8.7% in nuclear, 13.0% in mitochondrial and 4.3% in secretory vesicles in geese (MA *et al.* 2015). The predicted subcellular location of goose ODC protein was 65.2% cytoplasmic, 21.7% nuclear and 13.0% mitochondrial. Our data showed that the subcellular location of goose AZIN2 protein was 47.8% cytoplasmic, 26.1% nuclear, 17.4% mitochondrial, 4.3% cytoskeletal and 4.3% plasma membrane.

A previous study showed that goose *AZIN1* mRNA was expressed in all examined tissues (MA *et al.* 2015). In this study, the observed mRNA expression levels of *AZIN2* in the heart, adrenal glands, breast muscles, thigh muscles and pineal gland in geese were lower than the detection limits of qRT-PCR used. This suggests that *AZIN1* is ubiquitously expressed and *AZIN2* shows restricted expression in goose tissues. In mammals, studies have revealed that *AZIN2* was mainly expressed in testis and brain (LOPEZ-CONTRERAS *et al.* 2010), but not evenly expressed in all types of cells. *AZIN2* mRNA expression is mainly observed in the testicular germinal haploid cells (LOPEZ-CONTRERAS *et al.* 2009a), whereas in the murine brain *AZIN2* appears to be mainly localized in motor and sensory nucleus, hippocampus and some cerebellar areas (LOPEZ-CONTRERAS *et al.* 2010; RAMOS-MOLINA *et al.* 2012). These results suggest that *AZIN2* has a more restricted pattern of expression than *AZIN1* in both bird and mammalian tissues (LOPEZ-CONTRERAS *et al.* 2010). AZIN2 is exclusively expressed in adrenal medulla of adrenal glands that plays a role in the bio-

synthesis and secretion of catecholamines (LOPEZ-CONTRERAS *et al.* 2009b; LOPEZ-GARCIA *et al.* 2013). However, in present study, the mRNA expression of *AZIN2* was not found in adrenal glands in the goose. The mRNA expression of *AZIN2* in different cells of adrenal glands remains to be measured in birds. Studies have suggested that robust expression of *AZIN2* in the brain is distributed along neural axons and dendrites in a granular or vesicular pattern (MAKITIE *et al.* 2010; RASILA *et al.* 2016). High expression of AZIN2 was also found in human cerebellum (RASILA *et al.* 2016). In this study, high mRNA expression levels of *AZIN2* were observed in cerebrum, cerebellum and hypothalamus tissues in the goose. It is well known that AZIN2 is an inhibitor of OAZ. Thus, it is conceivable that AZIN2 is involved in regulating polyamine homeostasis in the brain. Changes in the expression and activity of different polyamine metabolic enzymes, as well as alternations in polyamine levels, have been associated to different brain insults such as cerebral ischemia and some mental disorders (LI *et al.* 2007; FIORI & TURECKI 2008; LOPEZ-CONTRERAS *et al.* 2010). Taken together, strong AZIN2 expression in the brain suggests that this protein may play important roles in modulating physiological functions of the brain in both mammals and birds, although the actions and mechanisms need to be clarified.

Polyamine synthesis, under endocrine influence, appears necessary for function and differentiation of the somatic cell component of the ovary (THYSSEN *et al.* 2002; FERNANDES *et al.* 2017; QIU *et al.* 2017). Treatment of immature female mice with DFMO (an irreversible inhibitor of ODC) inhibits ovarian growth, antral follicle formation, and the onset of puberty (BASTIDA *et al.* 2005; QIU *et al.* 2017). As commented before, AZIN can restore ODC activity by forming a tight complex with OAZ, thereby releasing ODC from the ODC-OAZ complex (QIU *et al*. 2017). Taking into consideration polyamines and ODC action as a regulator of ovarian function, it is possible that AZINs should play important roles in regulating follicular development. *AZIN1* mRNA expression gradually increased during follicular development and was significantly higher in POF1 follicle than other ovarian follicles. This suggests that AZIN1 plays a key role in follicular development (MA *et al.* 2015). In the present study, significant differences were not detected among the SWF, SYF and F5-F2 follicles, whereas the mRNA expression level of *AZIN2* was significantly higher in the F1 follicle than other follicles. Recent studies suggest that AZINs might be important molecules for modulating cell proliferation and oncogenesis through both the polyamine pathway and additional mediators (LOPEZ-CONTRERAS *et al.* 2010; LOPEZ-GARCIA *et al.* 2013; QIU *et al.* 2017). Taken together, high expression of *AZIN1* in the POF1 follicle and of *AZIN2* in the F1 follicle indicate that AZINs may play an important role in modulating follicular development and ovulation. The actions and molecular mechanisms responsible for these functions remain to be determined.

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Author Contributions

Research concept and design: B.K., D.M.J.; Collection and/or assembly of data: B.K., T.D., X.X.W.; Data analysis and interpretation: B.K., Z.Y.C., Z.X.Y., D.M.J.; Writing the article: B.K., D.M.J.; Critical revision of the article: B.K, T.D., Z.Y.C., X.X.W., Z.X.Y., D.M.J.; Final approval of article: D.M.J.

Conflict of Interest

The authors declare that they have no conflict of interest.

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