Chicken *GLUT4* undergoes complex alternative splicing events and its expression in striated muscle changes dramatically during development

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ABSTRACT Glucose transporter protein 4 (***GLUT4***) plays an important role in regulating insulin-mediated glucose homeostasis in mammals. Until now, studies on *GLUT4* have focused on mammals mostly, while chicken *GLUT4* has been rarely investigated. In this study, chicken *GLUT4* mRNA sequences were obtained by combining conventional amplification, 5′- and 3′- rapid amplification of cDNA ends technique (**RACE**), then bioinformatics analysis on its genomic structure, splicing pattern, subcellular localization prediction and homologous comparisons were carried out. In addition, the distribution of *GLUT4* was detected by RT-qPCR in bird′s liver and striated muscles (cardiac muscle, pectoralis and leg muscle) at different ages, including embryonic day 14 (**E14**), E19, 7-day-old (**D7**), D21 and D49 (n = 3−4). Results showed that chicken *GLUT4* gene produced at least 14 transcripts (GenBank accession No: OP491293-OP491306) through alternative splicing and polyadenylation, which predicted encoding 12 types of amino acid (**AA**) sequences (with length ranged from 65 AA to 519 AA). These proteins contain typical major facilitator superfamily domain of glucose transporters with length variations, sharing a common sequence of 59 AA, and were predicted to have distinct subcellular localization. The dominant transcript (named as T1) consists of 11 exons with an open reading frame being predicted encoding 519 AA. In addition, analyzing on the spatiotemporal expression of chicken *GLUT4* showed it dominantly expressed in pectoralis, leg muscles and cardiac muscle, and the mRNA level of chicken *GLUT4* dramatically fluctuated with birds′ development in cardiac muscle, pectoralis and leg muscles, with the level at D21 significantly higher than that at E14, E19, and D49 ($P < 0.05$). These data indicated that chicken *GLUT4* undergoes complex alternative splicing events, and *GLUT4* expression level in striated muscle was subjected to dynamic regulation with birds′ development. Results indicate these isoforms may play overlapping and distinct roles in chicken.

Key words: *GLUT4*, gene cloning, bioinformatics analysis, expression

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INTRODUCTION

Glucose is the most essential metabolic fuel for living organism (Yea et al., 2009), and glucose transporter (**GLUT**) protein family plays a rate-limiting role in glucose metabolism of diverse organisms from microbes to humans (Farese et al., 2007; Sun et al., 2012). As a member of GLUT protein family, GLUT4 (also known as SLC2A4) is the major insulin-inducible and contraction-stimulated glucose transporter and plays a key role in glucose homeostasis through controlling glucose uptake and transport into fat, muscle tissues and cardiomyocytes in mammals (Uldry and Thorens, 2004; Richter and Hargreaves, 2013; Klip et al., 2019). Under basal (low insulin) conditions, GLUT4 resides primarily in intracellular compartments, while when circulating insulin levels rise after the ingestion of the carbohydrate meal, GLUT4 translocation from intracellular vesicles to the plasma membrane and glucose transport in these tissues are stimulated rapidly (Jaldin-Fincati et al., 2017; Fazakerley et al., 2022). A defect in insulin regulation of GLUT4 trafficking could contribute to whole-body insulin resistance and complex metabolic diseases such as type 2 diabetes in humans (Mueckler and Thorens, 2013; Klip et al., 2019; Beckerman et al., 2021). Following the first report of human *GLUT4* in 1988 (James et al., 1988), *GLUT4* was successively reported in other species, such as rat (Birnbaum, 1989), mouse (Kaestner et al., 1989), brown trout (Planas et al., 2000) and common carp (Yang et al., 2021).

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Birds, have distinct glucose metabolism feature from mammals. They are more insulin-resistant and have unusually higher blood glucose level (twice as mammals') for life (Akiba et al., 1999; Braun and Sweazea, 2008). Evidence showed that exogenous insulin rapidly decreased chicken blood glucose concentration (Sumners et al., 2014; Ji et al., 2020) and improved the glucose uptake in skeletal muscle (Tokushima et al., 2005), which suggested birds possess similar regulation mechanism on glucose homeostasis as presented in mammals and may possess the gene homologous to mammals′ *GLUT4*. It aroused the interest of many researchers (including our lab) on conducting the study of glucose transporters in chickens and other birds. However, the cloning and molecular functions of bird *GLUT4* are unclear so far, even it has become a controversial topic in a long time for whether *GLUT4* gene be present in chicken genome (Carver et al., 2001; Byers et al., 2017; Xiong and Lei, 2021). Several researchers supposed that birds have high blood glucose levels and low sensitivity to insulin due to the absence of *GLUT4* (Satoh, 2021), and other glucose transporters such as *GLUT12* may compensate for the loss of *GLUT4* in birds (Xiong and Lei, 2021). Whereas some studies reported that GLUT4 was detected in skeletal muscle of duck with rat GLUT4 antibody, and it showed molecular and functional homologies with the mammalian GLUT4 protein (Thomas-Delloye et al., 1999). Shi et al. detected weak GLUT4 protein expression from E18 to E20 in chicken with mouse GLUT4 antibody (Shi et al., 2014). Unlike mammals, bird′s genome contains abundant micro-chromosomes, where there are complex genome structure and high GC content. Therefore, we presumed that birds′ *GLUT4* may be buried in the genome due to the incomplete reference genome/complex genomic structure (Yin et al., 2019).

Blast searching with human and mouse *GLUT4* in NCBI database (https://www.ncbi.nlm.nih.gov/) showed that the predicted low-quality *GLUT4*-like gene was annotated in the refence genome of various birds, such as chicken (*Gallus gallus*, XM_025145961.1), Japanese quail (*Coturnix japonica*, XM_015850416), rock ptarmigan (*Lagopus muta*, XM_048933136) and turkey (*Meleagris gallopavo*, XM_010727133). In Ensembl database (http://asia.ensembl.org/index.html, GRCg6a), chicken *GLUT4*-like gene was named as ENSGALG00000049504 (locating at chr.31), containing three transcript variants (ENSGALT00000106036, ENSGALT00000101195 and ENSGALT00000093891, ENSGALT00000093891 was removed in 2022). In addition, manual synteny analysis suggested that they should be *GLUT4* gene in birds.

In this context, we hypothesized that chicken *GLUT4* may undergone complex splice events to produce abundant transcripts, and be dynamically regulated in tissue- and development- specific manner. With the development of strand-specific high-throughput sequencing technology, it revealed that there were abundant transcripts locating on the opposite of target transcribed strand, while strand-specific Rapid amplification of cDNA ends (**RACE**) technique could effectively avoid the ambiguity from another strand (Pal, 2022). Therefore, this study aimed to 1) clone and identify chicken *GLUT4* transcript variants and its structure features by RACE technique and bioinformatic tools, and 2) to investigate its expression profile in the dominant expressed tissues at different development stages to reveal the potential role of *GLUT4* in chicken. This study will promote better understanding of glucose homeostasis and delineate the mechanism of regulated glucose transport in chicken.

MATERIALS AND METHODS

Experimental Animals

In the experiment, 100 chick embryos of Arbor Acres broilers were hatched in biochemical incubator with temperature of 37 ± 0.5°C and humidity of 55 to 60%. After hatching, they were raised with free access to water and a conventional balanced diet. Diets were formulated according to the Chicken feeding standard (NY/T33-2004) until D49. After overnight fasting, birds were euthanatized by cervical dislocation. The tissue samples including heart, liver, lung, kidney, pectoralis, leg muscle, glandular stomach, brain, and abdominal fat were collected from different development stages (E14, E19, D7, D21, and D49) of birds (n = 3−4). All samples were snap-frozen in liquid nitrogen and transferred to −80°C for RNA extraction. All procedures were approved by Henan Agricultural University Institutional Animal Care and Use Committee (No. HNND20191201).

RNA Extraction and First-Strand Complementary DNA (cDNA) Synthesis

Total RNA was extracted from tissues with the TransZol RNA isolation kit according to the instruction (TransGen Biotech Co. Ltd, Beijing, China). The concentration and purity of RNA were determined by UV-visible spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The integrity of RNA was detected by 1% agarose gel electrophoresis. Subsequently, 1,000 ng valid RNA samples were reverse-transcribed to synthesize the first-strand cDNA in 20 μL reaction system with a HiScript II 1st Strand cDNA Synthesis Kit (plus gDNA wiper) (Vazyme Biotech Co. Ltd, Nanjing, China).

Cloning of GLUT4

The cloning of chicken *GLUT4* was finished with total RNA from broiler's pectoralis at D21. First, a conventional PCR amplification and PCR sequencing were performed based on chicken *GLUT4*-like sequence (XM_025145961.1) with primer set G1 (Table 1). Then based on the sequences obtained by primer set G1 and the common sequences of 2 versions in the NCBI database (XM_025145961.1 and XM_040657202.1), the overlapping primers (5′/3′ GSP and 5′/3′ NGSP) were

designed for 5′- and 3′-RACE (Table 1). The 5′- and 3′-RACE were conducted according to the manufacturer′s instructions (SMARTer RACE 5′/3′ Kit, Takara, Kyoto, Japan). Briefly, 20 μL 5′- or 3′- RACE-ready-cDNA were synthesized from 1,000 ng total RNA with the 5′- or 3′- CDS Primer A respectively. Next, the first round of 5′- or 3′-RACE amplification was performed by using 5′- or 3′-RACE-ready-cDNA template with UPM-L and GSP, then first round products of 5′- or 3′-RACE amplification were diluted at 1:50 and used as templates for nested PCR reactions with UPM-S and NGSP. The UPM-L and UPM-S primers were provided in the kit. The first round amplifications were performed with 50 μL reaction mixtures containing 2.5 μL of 5′- or 3′-RACE-ready-cDNA template, using the program: 95°C for 5 min; followed by 20 cycles of 95°C for 30 s, 70°C to 60°C for 30 s (each cycle minus 0.5°C), 72°C for 1 min; then 25 cycles of 95°C for 30 s, 62°C 30 s, 72°C for 1 min; and a final extension step of 72°C for 10 min. The nested PCR reactions were performed with 50 μL reaction mixtures containing 5 μL of template under the following condition: incubation at 95°C for 5 min; 25 cycles at 95° C for 30 s, 66°C for 30 s, 72°C for 2 min; and a final extension step of 72°C for 5 min. The nested PCR products of the 5′-RACE and 3′-RACE were purified with the gel extraction kit (Vazyme Biotech Co. Ltd, China), and then cloned into the TOPO vector (Vazyme Biotech Co. Ltd, Nanjing, China), followed by transformed into Escherichia coli (***E. coli***) strain Trans5*α* (Tsingke Biotechnology Co., Ltd, Beijing, China). The positive clones were conducted Sanger sequencing. Primers were designed with online primer-blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Shanghai Bioengineering Co., Ltd (Table 1).

Bioinformatics Analysis

The genomic feature of transcripts was analyzed by blast searching chicken genomic database in NCBI platform (GRCg7b). The exon/intron boundaries of chicken *GLUT4* transcripts were further determined by combining manual analysis based on GT-AG rule. The amino acid sequences were predicted based on the maximum open reading frame (**ORF**) of each transcript by DNAMAN software. NCBI conserved domains tool was used for the annotation of sequences with the locations of conserved protein domain footprints, and the functional sites/motifs inferred from these footprints (Lu et al., 2020). PSORT Prediction software was used to predict the subcellular localization (https://psort.hgc.jp/form2.html). Multiple alignments for amino acid sequences were performed by Meg-Align (DNASTAR, Madison, WI) and DNAMAN software. A phylogenetic tree was further constructed based on the Neighbor-Joining method with a bootstrap coefficient of 1000 by MEGA7 (Felsenstein, 1985; Saitou and Nei, 1987). The genomic synteny of chicken *GLUT4* was analyzed manually after downloading the corresponding genome sequence of different species. The tertiary structure of chicken GLUT4 protein was predicted by SWISS-MODEL (https://swissmodel.expasy.org/interactive).

Analysis of Gene Expression Profiles

RT-qPCR was used to determine the expression of total *GLUT4* gene in chicken on BioRad CFX96 (Bio-Rad, Hercules, CA), by primer set G5 located at the common region of transcript isoforms (Table 1). It was performed in 10 μL reaction system: 5 μL of 2 × SYBR Premix ExTaqTM (Vazyme Biotech Co. Ltd, China), 0.2 μL (0.1 μM) of upstream and downstream primers (Table 1), 1 μL of tissue cDNA and 3.6 μL of ddH_2O. The cycling conditions were as follows: 95°C for 30 s, and 40 cycles of 95°C for 10 s, 60°C for 30 s, and *β-actin* was used as a reference to normalize the expression of target gene. The amplification without template cDNA was taken as the negative control. All experiments were performed with 3 technical replicates and at least three

Table 1. Primers used in this work.

Primer name	Primer sequences (5′-3′)	Expected length(bp)
5′ GSP	CCCCAGGATGCCGATGACGACGGCCA	
5′ NGSP	AGGCGCCGAGCAGGAACCGACCAAT	
3′ GSP	TGGCTGAGCGCCTCGGCAGGAAGCA	
3′ NGSP	CATCATTGGTCGGTTCCTGCTCGGC	
UPM-L	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
UPM-S	CTAATACGACTCACTATAGGGC	
G1	F: TTCCAGCAGATCCAGAATGAGG	910
	R: CCGTGGAGTAATAGAAGATCGCA	
G2	F: GATCCAGAATGAGGAGGAGGACG	888
	R: AAGATCGCATTGATGCCGGA	
G3	F: CCTCACGCTCCTATTGGGTC	659
	R: AGAAAGCGAAGAGCCACGAA	
G4	F: ATCCAGAATGAGGAGGAGGATGC	517
	R: CGACGGCCAATTGATGCAG	
G5	F: CATGATTGCCACCAACGCC	103
	R: GAGCAGGAACCGACCAATGA	
β-actin	F: GTCCACCGCAAATGCTTCTAA	78
	R: TGCGCATTTATGGGTTTTGTT	

Note: F: forward primer; R: reverse primer.

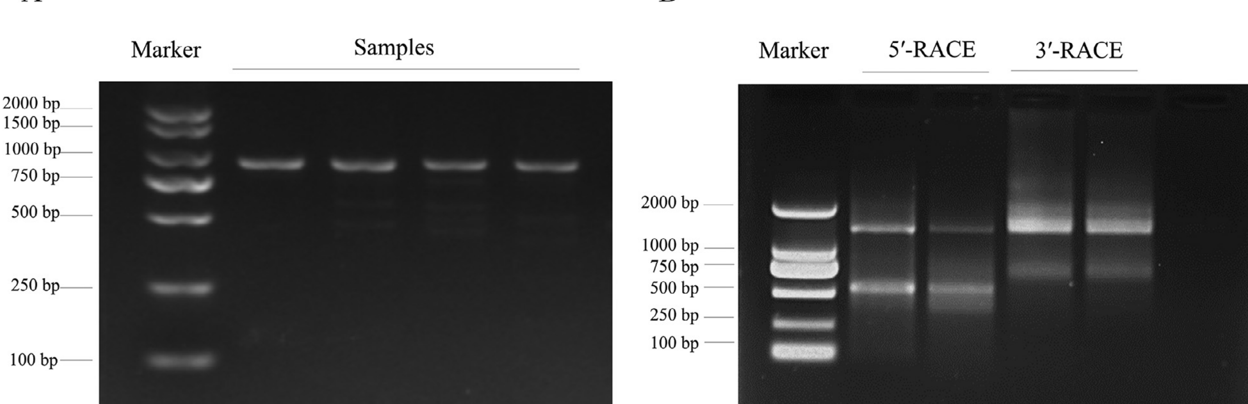

Figure 1. Cloning of chicken *GLUT4*. (A) PCR amplification results using primer set G1. (B) 5′-RACE and 3′-RACE results.

biological replicates. Relative expression level of *GLUT4* was normalized to *β-actin* and calculated using the $2^{-\Delta\Delta Ct}$ method (Rao et al., 2013).

Statistical Analysis

RT-qPCR data were analyzed with SPSS26.0 software. Differences were analyzed by one-way analysis of variance (**ANOVA**), followed with a Duncan or Games-Howell post-hoc testing. Data were represented as means ± standard error of mean (**SEM**). $P < 0.05$ was considered significant.

RESULTS

Abundant Alternative Splicing Forms of Chicken GLUT4

An about 900 bp product was gained with primer set G1 by conventional PCR amplification, direct PCR sequencing confirmed that the sequence was same as the predicted chicken *GLUT4*-like sequence (XM_025145961, Figure 1A). Based on the gained sequence by G1 primer set, 5′- and 3′-RACE were performed with the overlapping gene-specific primers (Figure 1B, Figure 2A). After the nested amplification, the 5′-RACE produced 2 products of approximately 1,500 bp and 500 bp (lanes 2 and 3, Figure 1B), whereas the 3′-RACE produced one strong band at approximately 1500 bp and a weaker band about 600 bp in length (lanes 4 and 5, Figure 1B). Similar results were obtained with different annealing template (data not shown). We cloned the 5′- and 3′- RACE products into the vector, and identified multiple clones by Sanger sequencing. Finally, 35 positive clones from 5′-RACE and 12 positive clones from 3′-RACE were successfully sequenced in total. The full transcripts of chicken *GLUT4* were obtained by assembling the 5′- and 3′-RACE sequences. The most region of the main transcript (T1 isoform) were further confirmed by combining the conventional PCR amplification and direct Sanger

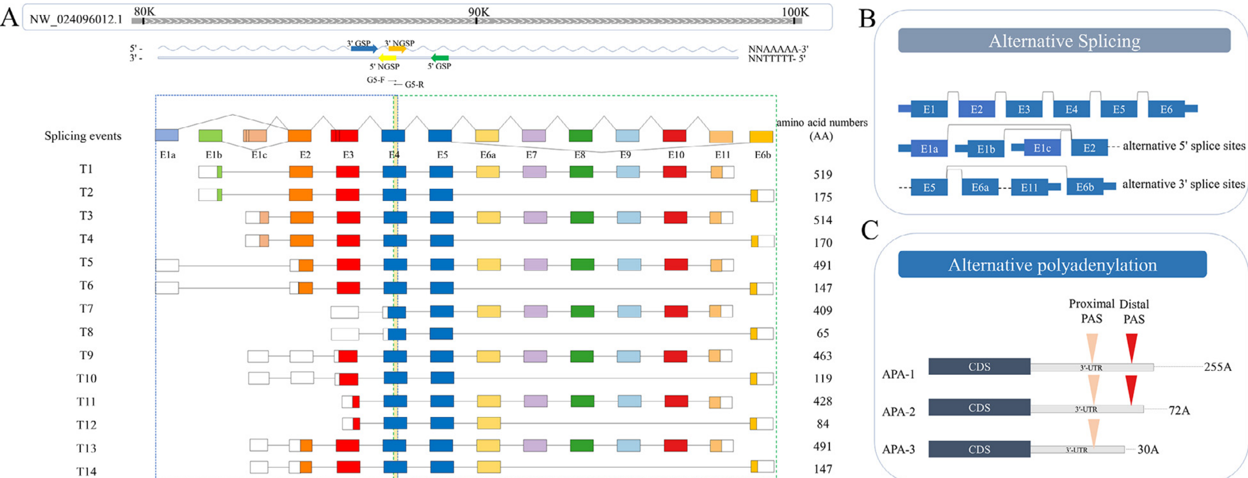

Figure 2. Graph visualization of alternative splicing and polyadenylation patterns for chicken *GLUT4* gene. (A) The genomic structure of fourteen splice isoforms identified by 5′-RACE (in blue dash box) and 3′-RACE (in green dash box). The corresponding genomic region spanning chicken *GLUT4* and primers used in this work were presented (top). Coding exons were expressed as colored box, and 5′-UTR and 3′-UTR were expressed as white box in the schematic diagram of splicing event. The fine line in exon E1c and E3 represented the alternative start position as presented in Table 3. (B) A brief description of the alternative splicing phenomenon. (C) Detected alternative polyadenylation events from the 3′-UTR of isoforms containing E11 (presented in Figure 1A). The ploy (A) length was presented.

Table 2. Information of different chicken *GLUT4* transcript variants.

Transcripts name	Full length (bp)	5′-UTR length (bp)	3′-UTR length (bp)	CDS length (bp)	Predicted amino acid numbers (AA)
T1*	2659−2943	898	202−486	519	1560
T2	1693	898	266	175	528
T3*	1786−2070	40	202−486	514	1545
T4	820	40	266	170	513
T5*	1757−2041	80	202−486	491	1476
T6	791	80	266	147	444
T7*	1459−1743	28	202−486	409	1230
T8	493	28	266	65	198
T9*	1775−2059	182	202−486	463	1392
T10	809	182	266	119	360
T11*	1580−1864	92	202−486	428	1287
T12	614	92	266	84	255
T13*	1763−2047	86	202−486	491	1476
T14	797	86	266	147	444

Note: For the transcript (those containing exon E11 as mentioned in Figure 2A) marked with "*", the 3′-UTR length varied depending on the type of APA selected (APA-1, APA-2, and APA-3) as presented in Figure S3.

sequencing with G2 to G4 primer sets. However, abundant alternative isoforms of chicken *GLUT4* were identified through clone sequencing the 5′- and 3′- RACE products. Seven transcripts were identified from the 5′-RACE products and 2 transcripts were identified from the 3′-RACE products (Figure 1A). After assembling the sequences gained by 5′-RACE and 3′-RACE, fourteen transcript isoforms (T1-T14) of chicken *GLUT4* gene were obtained (GenBank accession No: OP491293-OP491306), which varied at the 5′-end and 3′-end of sequence (Figure 2A, Figure S2, Table 2). It was observed that the identified transcript variants were mainly caused by two kinds of alternative splicing (**AS**) pattern: alternative first exon and alternative last exon (Figure 2B). In addition, three alternative polyadenylation (**APA**) events were detected from the 3′-UTR of the isoforms containing E11 (as presented in Figure 2C) by 3′-RACE, which contain alternative polyadenylation signal (**PAS, AATAAA**) with varied lengths in Poly (A) tail (Figure 2C, Figure S3). These transcripts could effectively map to chicken unplaced genomic scaffold (NW_024096012.1, GRCg7b), spanning about 17.3 kb genomic region (Figure 2A, Table 3), where low quality chicken *GLUT4*-like gene (LOC107049937) just located. The intron-exon boundaries of these transcript variants abide by GT-AG splicing rule except E6b (Table 3).

AS Transcripts Predicted Encoding Proteins With Variation in Length

The 14 transcripts isoforms of chicken *GLUT4* were predicted encoding 12 types of AA sequences with length ranging from 65 AA to 519 AA (named as PT1-PT14, Figure 3A and Figure S2). Transcript T5 and T13, T6 and T14 predicted encoding the same AA sequence respectively. The APA variation in the 3′-UTR does not affect the predicted ORF. In spite the dramatic variation in length, all 12 types of predicted proteins (PT1-PT14) possess a 59 AA identical sequence (Figure 3A), which is a conservative element unique for GLUT4 protein across species, sharing 71% and 68% similarity with the corresponding region of mouse (NP_033230.2) and human (NP_001033.1) (data not shown). These proteins were predicted to contain a Class I GLUTs of the major facilitator superfamily (**MFS**) structural domain (cd17431) with length variation (Figure 3A), and have distinct subcellular localization in cell (Figure 3B). Comparing with the sequence changes in 5′-end, the change in the 3′-end of chicken GLUT4 could more strongly affect the predicted subcellular localization of GLUT4. For example, the PT1 was predicted to be mainly located in the cell plasma membrane, while PT2 mainly existed in the vacuolar and endoplasmic reticulum, in spite they possessed the same 5′-end sequence (Figure 3B and Table S1).

T1 Transcript was the Predominant Splice Forms of Chicken GLUT4

Through combining counting the positive clones of RACE products with the confirmation by conventional

Table 3. The genomic structure of chicken *GLUT4* transcript variants.

	Genome region	Start	Stop	Exon-intron boundary
E1a*	81912−81945	81912	81945	GT-AG
E1b*	82615−83553	82615	83553	GT-AG
E1c*	85676−85738	85676[⊛1]	85738	GT-AG
E2	86942−87061	86942	87061	GT-AG
E3*	87809−87969	87809[⊛2]	87969	GT-AG
E4	88711−88835	88711	88835	GT-AG
E5	89734−89849	89734	89849	GT-AG[6a]/GG-AG[6b]
E6a	91272−91431	91272	91431	GT-AG
E7	93742−93923	93742	93923	GT-AG
E8	94859−94963	94859	94963	GT-AG
E9	96135−96236	96135	96236	GT-AG
E10	96930−97130	96930	97130	GT-AG
E11[#]	98608−99087	98608	99087[⊛3]	
E6b[#]	99074−99262	99074	99262	

Note: * represents the alternative first exon,
[#]represents the alternative last exon.
[⊛1]represents alternative starting position in E1c (85676, 85686 or 85699);
[⊛2]represents alternative starting position (87809, 87831 or 87952);
[⊛3]represents alternative stopping position in E11 (99027, 99072 or 99087), detail information shown in Figure S3. The version of chicken *GLUT4* genomic sequence was NW_024096012.1, GRCg7b from NCBI database.

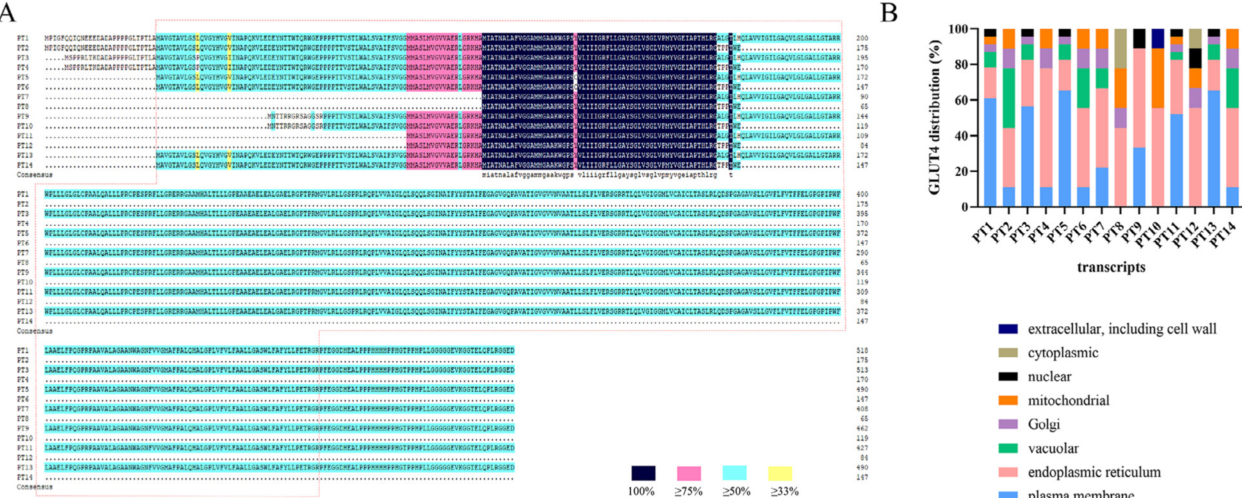

Figure 3. (A) Multiple alignment and (B) subcellular localization prediction for the predicted proteins of the corresponding *GLUT4* transcript variants in chicken. The sequence in red dashed box represents the possible Class 1 glucose transporters (GLUTs) of the Major Facilitator Superfamily structural domain (cd17431).

PCR amplification/Sanger sequencing using several pairs of primers (G1-G4, Table 1), it showed that T1 was the predominant transcript of chicken *GLUT4* (Figure S1). T1 transcript contained 11 exons with an 898 bp 5′-UTR and a 3′-UTR with length varying from 202 bp to 486 bp (due to APA variation), and an ORF being predicted encoding a 519 AA protein (Figure 2A, Figure S2 and Table 2).

Homology Analysis of GLUT4 among Species

To determine the homologous relationship of GLUT4 among species, the amino acid sequences of GLUT4 from mammals, birds, and fish were downloaded from NCBI database (Table S2). It showed that chicken GLUT4 (PT1) possesses all the features essential for sugar transport: 12 membrane-spanning helices, conserved AA motifs important for sugar transport activity (Figure 4A) and intracellular NH2 and COOH termini (Figure S4). Comparing with human and mouse GLUT4 protein, chicken GLUT4 possesses a conservative N-terminal FQQI motif and dileucine motif (LL) of C-terminal domain unique for GLUT4, but the C-terminal domain TELEY showed very low conservation in chicken GLUT4 (Figure 4A).

Multiple alignment among species showed that mammalian GLUT4 protein possessed high sequence identity with each other (above 90%), chicken GLUT4 shared 84.5% homology with Japanese quail (*Coturnix japonica*), while only had about 58.2% homology with human

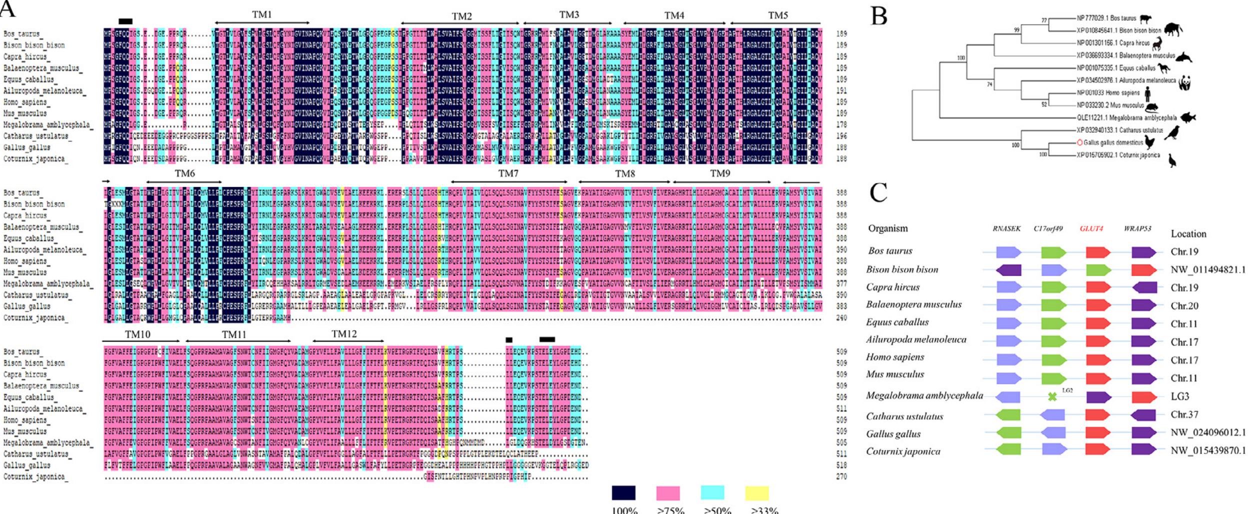

Figure 4. Homology analysis of GLUT4 proteins among species. (A) Multiple alignment of GLUT4 proteins among species. The 12 transmembrane domains (TM1-12) and conservative motifs (with black boxes) were marked on the top of corresponding region for GLUT4. (B) Constructed phylogenetic tree with chicken GLUT4 protein (PT1). Chicken GLUT4 was highlighted with a red circle. (C) Schematic of the chromosomic synteny encompassing *GLUT4*. Boxes in different color present different genes separately, and the gene with a green " × " represents C17orf49 on chromosome LG2 rather than chromosome LG3 in wuchang bream (*Megalobrama amblycephala*). The direction of different gene cassettes represents their transcriptional orientation. Here predicted protein of predominant transcript T1 (PT1) was used for homology analysis. Related protein sequence information was presented in Table S2.

(*Homo sapiens*) and house mouse (*Mus musculus*), which is lower than the homology between Wuchang bream (*Megalobrama amblycephala*) and any mammals (about 70%, Figure S5). Phylogenetic tree generated with chicken GLUT4 protein (PT1) effectively reflected the evolutionary relation of vertebrate, GLUT4 from mammals clustered together, while birds clustered to another branch (Figure 4B). It needs to be mentioned that the GLUT4 proteins were from the predicted mRNA sequences for that in Japanese quail (*Coturnix japonica*, XM_015850416.2, XP_015705902.1) and Swainson's thrush (*Catharus ustulatus*, XM_033084242, XP_032940133.1), which may reduce the sequence similarity with other species. Besides, the gene synteny was further analyzed, and it indicated that the synteny of the genomic region surrounding chicken *GLUT4* was highly conservative among birds, with the gene order in *C17orf49-RNASEK-GLUT4-WRAP53*. This chromosomal region was homologous to chr.19 in cattle (*Bos taurus*), chr.17 in human (*Homo sapiens*), chr.11 in house mouse (*Mus musculus*), and chr.17 in giant panda (*Ailuropoda melanoleuca*), etc. (Figure 4C). In addition, some differences in gene order and the transcription direction between birds and mammals were observed.

Spatial and Temporal Transcriptions of GLUT4

In attempt to elucidate the potentially physiological function of chicken *GLUT4*, a pair of primer G5, located at the common region of transcript isoforms (Figure 2A, Table 1), was designed to investigate the relative abundance of total *GLUT4* in chicken by RT-qPCR. It showed that chicken *GLUT4* mRNA extensively expressed in all detected tissues (Figure 5A). However, it exhibited distinct expression abundance among tissues, with the highest level in pectoralis, followed by leg muscle and heart at D21, and the level in pectoralis was significantly greater than that in the other detected tissues (about 2−2000-fold, $P < 0.001$, Figure 5A). Besides, *GLUT4* expression presented dynamic change with birds′ development and growth in chicken heart, liver, pectoralis and leg muscle (Figure 5B−E). In the heart, chicken *GLUT4* expression maintained a low level during E14 and E19, then rapidly increased during D7 to D21 (about 300-fold), and finally sharply dropped to embryonic level at D49 ($P < 0.001$, Figure 5B); On the other hand, total *GLUT4* expression showed a low level at E14, then increased gradually with development from E14 to D7 with a peak at D21, and then sharply dropped to the lowest level at D49 in pectoralis and leg muscle. The mRNA level of *GLUT4* at D21 was significantly higher than other time-points in pectoralis (about 3 to 1,000-fold, $P < 0.001$) and leg muscle (about 4−200-fold, $P < 0.01$, Figure 5B). *GLUT4* expression showed similar change trend in liver as in skeletal muscles with development, but the change was not significant ($P = 0.187$).

DISCUSSION

The key roles of *GLUT4* in glucose homeostasis and mechanism were abundantly documented in mammals. However, researches on bird′s *GLUT4* lagged behind for a long time. Based on some bioinformatics analyses, we supposed birds may possess the gene homologous to mammals′ *GLUT4* in their genome and conducted the cloning of chicken *GLUT4*. During this process, we found that the predicted low quality *GLUT4*-like mRNA sequences in chicken were updated several times in NCBI database (XM_025145961.1 in 2018, XM_040657202.1 in 2021 and XM_046906205.1 in 2022, now XM_025145961.1 and XM_040657202.1 have been removed as a results of standard genome annotation processing). In spite the predicted *GLUT4*-like mRNA sequences in 3 versions located at the same genomic region of chicken, they only shared about 560 bp common sequence (the common sequence overlapped exactly with our amplified fragment by G1 primers). In the newly updated version (in 2022, XM_046906205.1), the predicted *GLUT4*-like mRNA sequence was only 582 bp and predicted encoding a 193 AA protein in chicken, which had a big difference with the reported *GLUT4* in mammals. These showed the potential complex of *GLUT4* transcripts in chicken from a certain point of view.

To avoid the interference from another strand and get the full length of *GLUT4* transcripts by direct RACE technique, the overlapping 5′- and 3′-RACE primers based on the common sequence of XM_025145961.1 and XM_040657202.1 were designed. As predicted, chicken *GLUT4* transcripts were very complex. Cloning sequencing on 5′- and 3′-RACE products revealed the abundant diversity of amplified products, even it seemed there be some differences for each identified clone, which puzzled us. Nevertheless, almost all the identified sequence could effectively map to chicken genomic region containing the predicted *GLUT4*-like gene, which supported that we got the expected amplification products through 5′- and 3′-RACE technique. Finally, we identified at least 14 kinds of chicken *GLUT4* isoforms through assembling the 5′- and 3′-RACE products. In this period, Huttener et al reported a series "missing" genes (including *GLUT4, ALDOA, ENO3*, and *PYGM*) in birds (Huttener et al., 2021), and the chicken *GLUT4* sequence they got was just the predicted ORF of T1 transcript, which shed a light on our work. In their study, they also mentioned their hard work for getting bird′s *GLUT4* mRNA encoding the C-terminus. Combining with our experience, we speculated that the complex transcript phenomenon should be an important factor influencing on the research progress of *GLUT4* in bird.

The bird's *GLUT4* is a mystery for a long time, which may attribute to birds' complex genome structure in some degree. Unlike mammals, chicken nuclear genome contains a large of micro-chromosomes. Till now the chromosomal location was undetermined for bird′s *GLUT4*. It was mapped to chicken chr.31 in the former

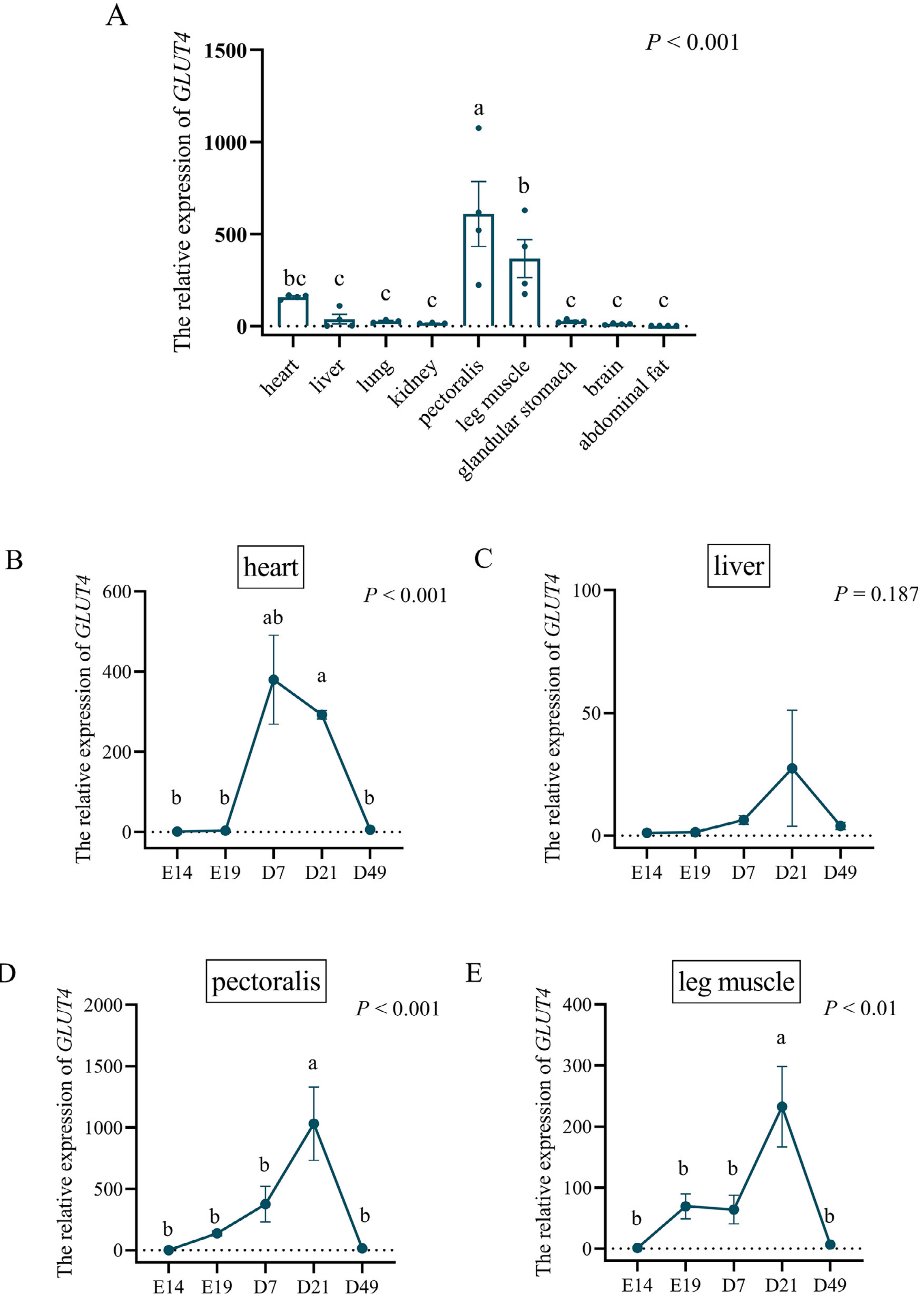

Figure 5. Spatial expression pattern of chicken total *GLUT4*. (A) The tissue expression pattern of chicken total *GLUT4* at D21. The transcript levels of total *GLUT4* in heart (B), liver (C), pectoralis (D) and leg muscle (E) at different development stages in chicken. mRNA abundance was quantified by RT-qPCR. *β-actin* was taken as the reference gene. Data were represented as mean ± SEM (n = 3−4). Different small letters above bars represent significant differences ($P < 0.05$).

version (NC_028740, GRCg6a), but now it was assigned to an unplaced genomic scaffold (NW_024096012.1, GRCg7b) in the newly updated version in 2022. Two versions of chicken genome sequencing project were from two distinct breeds [Red Jungle Fowl (GRCg6a) and maternal broiler (GRCg7b)] respectively. *GLUT4* transcripts identified could effectively map to two versions of chicken genomic sequences. We downloaded two versions of genomic sequence spanning chicken *GLUT4* [where no gap and poly (N) were

reported] from NCBI database [(NC_028740, GRCg6a) and (NW_024096012.1, GRCg7b)], and compared their similarity with online Global align software (across their entire span, Needleman-Wunsch; https://blast.ncbi.nlm.nih.gov/Blast.cgi). It was found these genomic sequences only shared 70% similarity, and where there were a rich variety of repetitive sequences. It reflected the rapid evolution of *GLUT4* genome in chicken. Huttener et al also mentioned chicken *GLUT4* protein undergo rapid evolution across species (Huttener et al., 2021). The rapid evolution of *GLUT4* in chicken may contribute to the complex transcript feature and even the specific glucose metabolism feature in birds (Li et al., 2021).

AS phenomenon is an extensive regulatory mechanism of gene expression that allows generation of more than one unique mRNA species from a single gene (Chen and Manley, 2009). In our research, by 5′- and 3′-RACE technique, it was observed bird's *GLUT4* generates abundant mRNAs transcript variants through AS by multiple mechanisms including alternative 5′-starts, alternative 3′-ends, exon skipping and mutually exclusive exons. In spite the great length variation (65−519 AA) for the predicted proteins of these isoforms (PT1−PT14, Table 2), they contained a variable- length MFS domain possessing the specific *GLUT4* conservative element. On the other hand, these proteins were predicted to have distinct subcellular localization, which means these proteins in bird's *GLUT4* locus may possess overlapping and separate function. Recently, more and more evidence demonstrated that AS contributes to proteome complexity and plays an important role (Dharmalingam et al., 2022). High-resolution mass spectrometry analyses revealed that about 37% of human protein-coding genes generates multiple protein isoforms (Kim et al., 2014). Meanwhile, the changes in 5′- and 3′-UTR of *GLUT4* in birds might function through affecting mRNA stability, localization, or translation (Baralle and Giudice, 2017). Therefore, further work is needed to identify the functional consequences for the identified splicing events of *GLUT4* transcript isoforms and potential proteins.

In addition, bird's *GLUT4* transcripts were observed undergoing APA regulation by 3′-RACE. Like AS, usage of alternative poly (A) sites allows a single gene to encode multiple mRNA transcripts. APA is emerging as a widespread mechanism used to control gene expression (Hardy and Norbury, 2016; Hong et al., 2020; Dharmalingam et al., 2022). In spite that APA regulation in the 3′-UTR don't affect the ORF of chicken *GLUT4*, it could modify the binding availability of microRNA or RNA (Di Giammartino et al., 2011; Wang et al., 2015), affect mRNA localization, translation and stability (Sun, et al., 2017). Even, it was reported that changes in the length of poly (A) tails had a strong correlation with translation efficiency (Subtelny et al., 2014). Therefore, the research needed to be further clarified on the potential function and mechanism of APA selection for bird's *GLUT4*.

In mammals, *GLUT4* highly expressed in tissues including skeletal muscles(Hansen et al., 1995; Beckerman et al., 2021), adipose tissue (Shepherd et al., 1993; Carvalho et al., 2005), and cardiac muscle (Slot et al., 1991; Abel et al., 1999). Furthermore, rat's *GLUT4* level was reported to be fiber type dependent in their skeletal muscles, where red skeletal muscle preferentially expressed more *GLUT4* mRNA as well as protein (Kern et al., 1990). Consistent with reports in mammals (James et al., 1988; Birnbaum, 1989), *GLUT4* mRNA in chicken predominantly expressed in striated muscles, such as skeletal muscles and heart, which indicated the predominant function of *GLUT4* in striated muscle is potentially conserved in mammals and birds. On the other hand, the low expression of chicken *GLUT4* in abdominal fat also reflected the specificity of *GLUT4* function in birds.

Analyzing on the spatio-temporal expression feature revealed that the relative abundance of *GLUT4* in birds distinctly depend on the developmental stage. In striated muscles and liver tissues, it was common to bird's *GLUT4* with a low level during hatching period and high expression at D21, which means bird's *GLUT4* mainly function after birth instead of embryonic stage. The low gene expression for birds' *GLUT4* in embryonic stage may be associated with low carbohydrate levels and utilization in egg yolk (van der Wagt et al., 2020). Lipid biosynthesis mainly occurs in the bird's liver (90 %) rather than the adipose tissue (Nematbakhsh et al., 2021). Our study found that although the change trend of *GLUT4* with growth in the liver of birds was similar with that in skeletal muscle, the level of *GLUT4* in the liver is low throughout the detected time-points, which reflected that *GLUT4* in bird's liver may not be as important as that in mammalian fat.

It is well known that Arbor Acres broiler is a kind of meat-type poultry. After long-term artificial selection, it grows very fast, whose individual body weight could achieve above 2.5 kg at D49 (Pan et al., 2005). In theory, more and more glucose need be transport to broilers' target tissues/organs by glucose carriers for meeting their fast muscle growth (Santalucia et al., 1992). While *GLUT4* level in bird's striated muscle dramatically dropped from the top to the bottom during D21 to D49 after undergoing the rapid increase before D21. On the other hand, *GLUT1* level decreased with birds' muscle embryogenesis (Carver et al., 2001). It suggested that other GLUT family members may play a complementary role during chicken *GLUT4* in low level. Till now, many members of GLUTs family have been identified (Mueckler and Thorens, 2013). *GLUT4* may function cooperatively with other GLUT members during bird's development and growth as that in mammals (Bowman et al., 2019). In rats, *GLUT4* and *GLUT1* level showed an inverse pattern of changes with development in insulin-sensitive tissues. *GLUT1* rapidly decreases in rat heart and skeletal muscle tissues soon after birth, while *GLUT4* increases during that time (Santalucia et al., 1992).

In addition, the complex transcript features hint that chicken *GLUT4* may undergo multiple modifications in the translational or posttranslational steps during development as reported in mammalian *GLUT4* (Gurley et al., 2016; Mori et al., 2019). Sometimes there was a dissociation during development between mRNA and protein levels for mammalian *GLUT4* (Santalucia et al., 1992; Zorzano et al., 1998). So, chicken *GLUT4* change in protein level needed to be further clarified.

In the past reports about chicken *GLUT4*, some researchers did not detect the *GLUT4* expression from chicken skeletal muscles or other tissues by western blotting even by northern blotting (Duclos et al., 1993; Carver et al., 2001; Seki et al., 2003), which may be related with the low specificity of the GLUT4 antibody/probe used and the dramatic change of GLUT4 level in birds during development in some degree.

Overall, limited research revealed that chicken *GLUT4* expression regulation seems be complex in birds. *GLUT4* in birds may undergo complex transcription level and post-transcription regulation as in mammals. Bird's *GLUT4* was drastically regulated in tissue- and development- dependent manner. Further study on the function and mechanism of chicken *GLUT4* transcription isoforms needed to be conducted.

CONCLUSION

In general, chicken *GLUT4* produces abundant transcript variants through multiple splicing mechanism, which were predicted encoding multiple proteins containing the MFS domain with length variation, and they were predicted to have distinct subcellular localization in cell. Analyzing on spatio-temporal expression revealed that bird's *GLUT4* predominantly expressed in striated muscles where the level dramatically fluctuated during the development and growth. Our findings may provide a solid foundation for future understanding the evolution of *GLUT4* and glucose metabolism functions in avian.

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DISCLOSURES

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.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102403.

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