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mTOR signaling pathway regulates embryonic development and rapid growth of triploid crucian carp

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ABSTRACT

Triploid crucian carp is a new breed of aquaculture with rapid growth characteristics. However, its growth regulation mechanism has not yet been elucidated. In this study, our results show that the expression levels of mTOR signaling pathway-related genes (*AKT1*, *AKT2*, *AKT3*, *mTOR*, *4E-BP1* and *S6K1*) are higher in the muscle and intestine tissues of triploid crucian carp than those of diploid red crucian carp. Embryos of triploid crucian carp exhibit a faster growth rate comparing to red crucian carp. And in muscle effect stage, the mRNA levels of mTOR signaling pathway related genes in triploid crucian carp are higher than red crucian carp, except for *AKT2*. Inhibition of *mTOR* activity with rapamycin leads to slower embryonic development and the eliminated expression of *mTOR* in the embryos of neurula, muscle effect, and eye pigmentation stages. Conversely, over-expression of the upstream gene *AKT2* can promote the development of zebrafish embryos and affect the expression of mTOR signaling pathway. These data highlight the significant role of mTOR signaling pathway in regulating fish embryonic development and rapid growth, and might offer new insights and avenues for exploring fish embryonic development, improving fish growth.

1. Introduction

Embryonic development and growth processes are crucial biological events, encompassing cell proliferation, differentiation, organogenesis, and growth regulation (Valet et al., 2022; Zhu and Thompson, 2019). Fish embryonic development and growth have generated significant interest among aquatic researchers (Fang et al., 2021). The State Key Laboratory of Developmental Biology of Freshwater Fishes at Hunan Normal University has been at the forefront of research in this area, and established a genetically stable, hermaphroditically fertile allotetraploid crucian carp (4 n = 200) population (Liu et al., 2001). Triploid crucian carp (3 n = 150) was produced by mating the male of allotetraploid with the female of crucian carp (*Carassius auratus cuvieri*). Triploid crucian carp was a type of sterile polyploid fish with the advantages of fast potential growth rate, high meat quality, strong resistance to adversity, and no interference with the growth and reproduction of fish resources (Liu et al., 2004), which provided excellent research materials for fish growth and development. Current research on triploid crucian carp mainly focused on sterility (Zhang et al., 2021), resistance (Liu et al., 2021; Liu et al., 2018) and nutrition (Tong et al., 2023; Xiao et al., 2022), but the molecular mechanism of its rapid growth has not been fully elucidated.

mTOR (mammalian target of rapamycin) signaling pathway has attached considerable attention in the field of cellular metabolism and growth regulation (Saxton and Sabatini, 2017; Schmelzle and Hall, 2000). As a critical intracellular signaling pathway, mTOR signaling pathway was involved in the regulation of essential biological processes such as cell proliferation, differentiation, metabolism, and survival (Kapahi et al., 2010; Lloyd et al., 2017). It exerted profound effects on cell and organismal growth and development by modulating key

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pathways including protein synthesis, energy metabolism, cell cycle, and autophagy (Liu and Sabatini, 2020). Additionally, mTOR signaling pathway interacted with various growth-related factors, metabolic pathways and signal transduction pathways to form a complex regulatory network (Ben-Sahra and Manning, 2017; Yu et al., 2008). Initially identified as the target protein of rapamycin, mTOR signaling pathway exerted its function through the formation of two distinct complexes: mTORC1 and mTORC2 (Laplante and Sabatini, 2013). mTORC1 primarily regulated protein synthesis, cell proliferation, and metabolic processes(Wang et al., 2022a), while mTORC2 modulated cytoskeletal remodeling, cell survival, and metabolic adaptation(Simcox and Lamming, 2022). The activity of mTORC1 was specifically inhibited by rapamycin, a natural compound with antifungal and antitumor properties (Grove et al., 1991; Kim and Sabatini, 2004; Kim et al., 2003). Ribosomal protein kinase (S6K), a serine/threonine kinase consisting of two catalytic subunits of different sizes (Holz et al., 2005), played a crucial role downstream of *mTOR* or *AKT* in promoting translation initiation complex assembly by phosphorylating multiple sites of ribosomal protein S6, thereby facilitating protein synthesis (Liu et al., 2010; Ma and Blenis, 2009). *mTOR* also phosphorylated multiple sites of *4E-BP1*, leading to its dissociation from the translation initiation factor eIF4E and subsequent binding to other translation initiation factors, ultimately promoting translation complex formation (Jefferies et al., 1997; Qin et al., 2016; Schwab et al., 1999; Sfakianos et al., 2018). The regulatory subunit PRAS40 negatively regulated *mTOR* activity, and its regulation was controlled by upstream *AKT* (Havel et al., 2015). *AKT*, also known as protein kinase B, was a vital protein kinase that promoted organismal viability and comprised three isoforms: *AKT1*, *AKT2* and *AKT3* (Brazil and Hemmings, 2001). AKT molecules possessed a central kinase subregion and an amino-terminal pleckstrin homology (PH) domain responsible for interactions with other proteins or lipids (Chu et al., 2020). In addition to directly regulating PRAS40, *AKT* also directly modulated the S6K protein kinase downstream of *mTOR* (Shao et al., 2006; Stephens et al., 1998).

In fish, inhibition of FXR could activate the PI_3K/AKT/mTOR pathway, and led to a decrease in lipolysis and an increase in lipogenesis, resulting in enhanced lipid accumulation (Xu et al., 2022). Wu et al. found that culturing juvenile Genetically Improved Farmed Tilapia (GIFT) (*Oreochromis niloticus*) at appropriate salinity level could enhance protein synthesis in fish by stimulating the mTOR signaling pathway, indicating the close relationship among the mTOR signaling pathway, protein synthesis, and fish growth (Wu et al., 2021). Additionally, researchers observed that dietary protein levels did not affect the mRNA levels of mTOR signaling pathway genes in juvenile blunt snout bream, but the stocking density significantly influenced the expression of mTOR signaling pathway genes (Yadata et al., 2020). However, there remained a gap in the studies of the role and regulatory mechanisms of the mTOR signaling pathway in the embryonic development and rapid growth of fish. Therefore, we used triploid crucian carp and diploid red crucian carp, which have obvious growth differences, as experimental objects, and also used the model organism zebrafish as auxiliary materials. We used gene cloning, qRT-PCR, western blot, rapamycin treatment, microinjection and other techniques to investigate the genes related to the mTOR signaling pathway (*AKT1*, *AKT2*, *AKT3*, *mTOR*, *4E-BP1*, and *S6K1*). Understanding the function of mTOR signaling pathway in triploid crucian carp can not only reveal the molecular mechanism of embryonic development and growth regulation, but also help to investigate the reproduction and breeding of different ploidy fish, which is the focus of this experiment.

2. Materials and methods

2.1. Fish

The experiments were carried out in the base of Engineering Research Center of Polyploid Fish Reproduction and Breeding of the State Education Ministry. The adult triploid crucian carp and red crucian carp (*C. auratus red var*) used for experimental sampling were fed thrice daily. Prior to sampling, they were fasted for 24 h (Cai et al., 2023). Ten one-year-old red crucian carp (body length of 10.98 ± 0.62 cm and body weight of 20.87 ± 1.28 g) and ten one-year-old triploid crucian carp (body length of 14.32 ± 0.76 cm and body weight of 49.86 ± 1.78 g) were selected for experimental sampling. After anesthetized, their muscle, intestine, heart, brain, kidney, gonad, and liver tissues were obtained and stored at − 80 ℃ for subsequent RNA and protein extraction.

Sexually mature parental fish were selected and obtaining fertilized eggs through artificially induced spawning (Fan et al., 2023; Wang et al., 2022b). Embryos from 7 different developmental stages (blastula, gastrula, neurula, muscle effect, eye pigmentation, body pigmentation, and hatching larva) were collected and stored at − 80 ℃ as materials for subsequent experiments.

2.2. Real-time quantitative PCR (qRT-PCR)

RNA extraction was performed using the RNAfast200 kit (Shanghai Feijie Biotech Co.), and the extracted RNA samples were analyzed for concentration and OD using a multifunctional enzyme marker. Reverse transcription of RNA into cDNA was carried out using the reverse transcription kit (TAKARA). Specific primers (Table 1) were designed for target genes, and *β-actin* as the reference gene. Melting curve analysis was performed to verify the specificity of each amplicon (Zhu et al., 2023). The amplification efficiency of all primers was higher than 98%. The relative expressions were determined using the $2^{-\Delta\Delta Ct}$ method and the data were analyzed and graphed using Graphed Prism 9 (Liu et al., 2021).

2.3. Western blot analysis

Protein extractions from muscle and intestinal tissues of red crucian carp and triploid crucian carp were performed using cell lysis buffer(Liu et al., 2013). Appropriate concentrations of sodium dodecyl sulfate-polyacrylamide gels were prepared for electrophoresis. After loading the protein samples, constant pressure electrophoresis was carried out, and the proteins were then transferred to nitrocellulose membrane. Subsequently, the nitrocellulose membranes were blocked in a blocking solution for approximately 1.5 h, followed by incubation of primary antibodies overnight on a shaker at 4 °C. The primary antibodies were purchased from Huabio and diluted as follows: anti-AKT (1:1000), anti-p-AKT (1:5000), anti-mTOR (1:1000), anti-p-mTOR (1:1000), anti-S6K1 (1:500), anti-p-S6K1 (1:1000), and anti-α-tubulin (1:1000). After washing the nitrocellulose membranes, anti-rabbit (Huabio) was diluted at 1:1000 and incubated based on the fact that all primary antibodies are rabbit recombinant monoclonal, and chemiluminescence was used for signal detection. Images were captured and analyzed for grayscale intensity using Image J software.

2.4. Inhibition of mTOR activity in triploid crucian carp and red crucian carp embryos with rapamycin

Holf solution was prepared by dissolving 0.1 g $CaCl_2$, 0.05 g KCl, and 3.5 g NaCl in distilled water to a final volume of 1 L. Next, the rapamycin (Pfizer Inc.) was dissolved in DMSO to prepare a 10 mM stock solution. Pre-experiments were conducted to determine the appropriate concentrations of rapamycin, and a concentration of 20 μM/L was selected for the main experiments. Embryos from the control group and the treated group were photographed, fixed, and collected for subsequent analysis.

2.5. Overexpression of AKT2

The pEGFP-N1 plasmid (4737 bp) was used as the vector, and the *AKT2* gene of yellow catfish (KX131158.1) was downloaded from the

Table 1
Primers used for qRT-PCR.

Genes	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	Product size (bp)
AKT1	CTGCCTGTCTCTATACCAGT	GCTCCAAACATCTCCGTTC	164
AKT2	ACACTAAAATACGCCTTCCAG	ACGACATCTTTAGAGTGCAGA	176
AKT3	CACAATCATATCTGGCAGGT	ATCATCTATCTTGGGCTGA	186
mTOR	CAAAGAGATGCAGAAGCCACA	CTCTCTCATACGCTCTCCC	178
4E-BP1	ATGACCGTAAGTTCCTGCTG	TTCAAACTGGGCATCATCC	200
S6K1	GACACAGCGCATACTAAAGCA	CCATAGAGATTTCAGCCAGGT	208
β-actin	TAAAGACCTGTATGCCAACACC	CAGACAGAGTATTTGCGCTCA	150

NCBI database (1440 bp). The first pair of primers was designed based on the cDNA sequence of the *AKT2* gene. The forward primer included the *Nhe*I enzyme cut site: AKT2-F: 5'-CTAGCTAGCATGAACGA-GATCAGCATCGTCAGAG-3', and the reverse primer included the HamdIII enzyme cut site design: AKT2-R: 5'- CCCAAGCTTCTCTCGCA-CACTGGCTGAGTAGGAG-3'. The second pair of primers was designed to verify the correctness of the constructed positive clone by targeting the *AKT2* gene and the vector. The PCR products were analyzed by agarose gel electrophoresis and purified. The vector plasmid and *AKT2* fragment were digested with *Nhe*I and HamdIII enzymes at 37 °C for 2–5 h. The *AKT2* fragment was then ligated to the digested vector using T4 phage DNA ligase at 16 °C for 12 h. The positive bacterial clone was cultured in a liquid medium supplemented with kanamycin, and the plasmids were extracted for microinjection into zebrafish using OME-GEN's Small Extraction Plasmid Kit. The pEGFP-N1-AKT2 recombinant plasmid was diluted to a concentration of 100 ng/μL for microinjection, and the empty vector was injected as the control group (NC).

3. Results

3.1. Expression of mTOR signaling pathway-related genes in adult tissues of triploid crucian carp and red crucian carp

To investigate the relationship between the mTOR signaling pathway and fish growth, we firstly examined the mRNA expression of mTOR signaling pathway-related genes in the muscle, intestine, heart, brain, kidney, gonad and liver tissues of triploid crucian carp and red crucian carp. The expression of *AKT1, AKT2, AKT3* genes in the muscle, intestine, heart, brain and kidney tissues of triploid crucian carp was higher than those of red crucian carp (Fig. 1A, B and C). The expression of

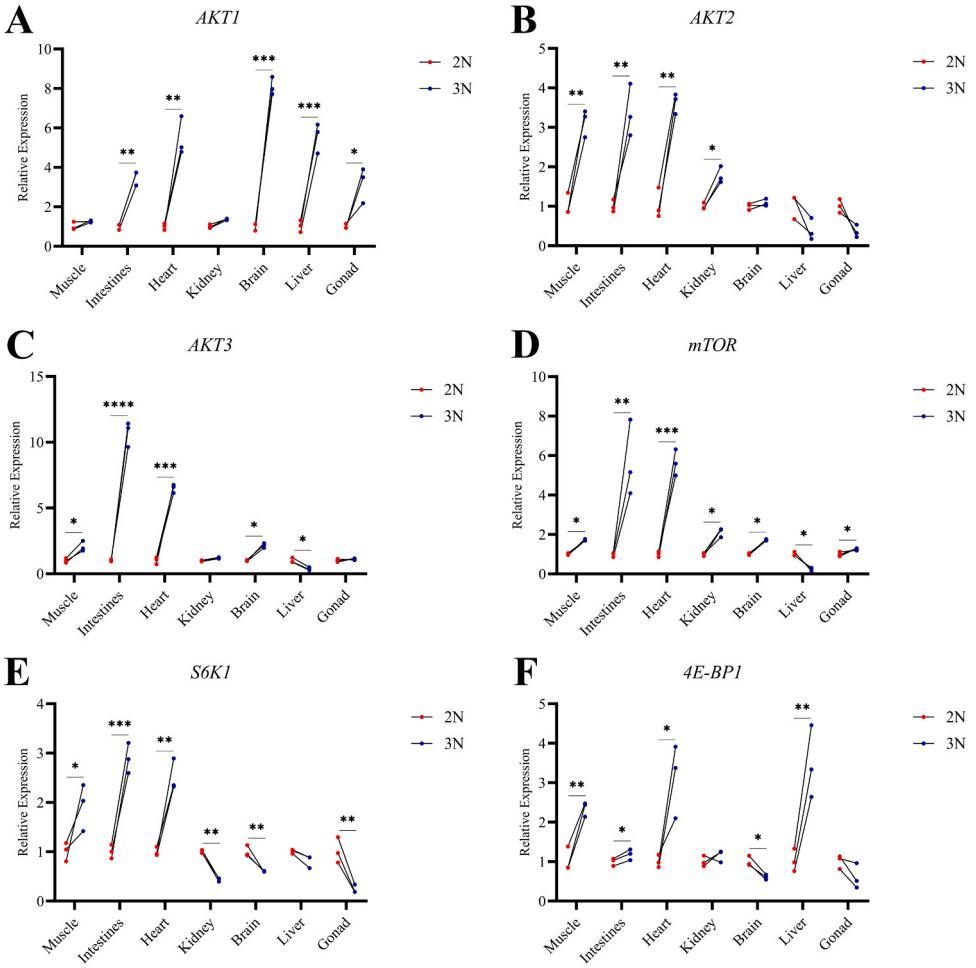

Fig. 1. Analysis of mRNA levels expression of mTOR signaling pathway-related genes in adult tissues of red crucian carp and triploid crucian carp. "2N" represents red carp; "3N" represents triploid crucian carp; * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$, **** represents $P < 0.0001$.

mTOR gene in muscle, intestine, heart, brain, kidney and gonad tissues of triploid crucian carp was significantly higher than that of red crucian carp, except for liver tissues (Fig. 1D). In addition, the expression of *S6K1* and *4E-BP1* genes in the muscle, intestine and heart of triploid crucian carp was significantly higher than those of red crucian carp (Fig. 1E and F).

We further examined the protein levels in muscle and intestinal tissues. As shown in Fig. 2, in muscle tissues, except for S6K1, the total protein levels of AKT and mTOR were notably elevated in triploid crucian carp compared to red crucian carp. Specifically, the mTOR protein level in triploid crucian carp was almost twice than that of red crucian carp. Furthermore, the phosphorylation levels of AKT, mTOR and S6K1 proteins were higher in triploid crucian carp than in red crucian carp (Fig. 2 A1-A3 and B1-B3). In intestinal tissues, the expressions of total protein levels of AKT, mTOR and S6K1 and phosphorylated AKT, mTOR and S6K1 were higher in triploid crucian carp than in red crucian carp, with significant differences except for total protein levels of S6K1 (Fig. 2 A1-A3 and C1-C3).

3.2. Expression analysis of mTOR signaling pathway-related genes in triploid crucian carp and red crucian carp embryos

To further explore the regulatory roles of mTOR signaling pathway in fish growth, we analyzed the expression patterns of mTOR signaling pathway-related genes in triploid crucian carp and red crucian carp during embryo development. Our results indicated that mTOR signaling pathway-related genes (*AKT1*, *AKT2*, *AKT3*, *mTOR*, *S6K1*, and *4E-BP1*) were expressed in various developmental stages of triploid crucian carp and red crucian carp embryos (Fig. 3). The expression of *AKT1* gene was significantly higher in all seven developmental stages of triploid crucian carp embryos compared to red crucian carp embryos (Fig. 3A). The expression of the *AKT2* gene was higher in triploid crucian carp than in red crucian carp before neurula stage, and lower from the muscle effect stage to the hatching larva stage (Fig. 3B). *AKT3* gene was significantly higher in triploid crucian carp embryos than in red crucian carp embryos at all stages except eye pigmentation and hatching larva stages (Fig. 3C). *mTOR* gene exhibited significantly higher expression in triploid crucian carp embryos in the gastrula, neurula, muscle effect, and body pigmentation stages (Fig. 3D). Moreover, the expression of *S6K1* gene was higher in triploid crucian carp than in red crucian carp, except for the gastrula and neurula stages (Fig. 3E). On the other hand, the expression of *4E-BP1* gene was significantly higher in triploid crucian carp embryos than in diploid red crucian carp during the muscle effect period, and there was no significant difference during the rest of the period (Fig. 3F).

3.3. Effect of rapamycin treatment on embryonic development of triploid crucian carp and red crucian carp

To examine the effect of *mTOR* activity on embryonic growth, we treated triploid crucian carp and red crucian carp embryos with rapamycin, an *mTOR* inhibitor (Um et al., 2019). Table 2 summarized the results of the rapamycin treatment. Under the same concentration of rapamycin incubation, 3 N treated group had lower mortality rate and malformation rate than 2 N treated group.

Observation results indicated that the growth rate of triploid crucian carp embryos was fast compared to the red crucian carp (Fig. 4A1-G1, A3-G3). When the embryos were treated with the *mTOR* activity inhibitors, a significant delay in growth rates was observed compared to the control groups in triploid crucian carp and red crucian carp

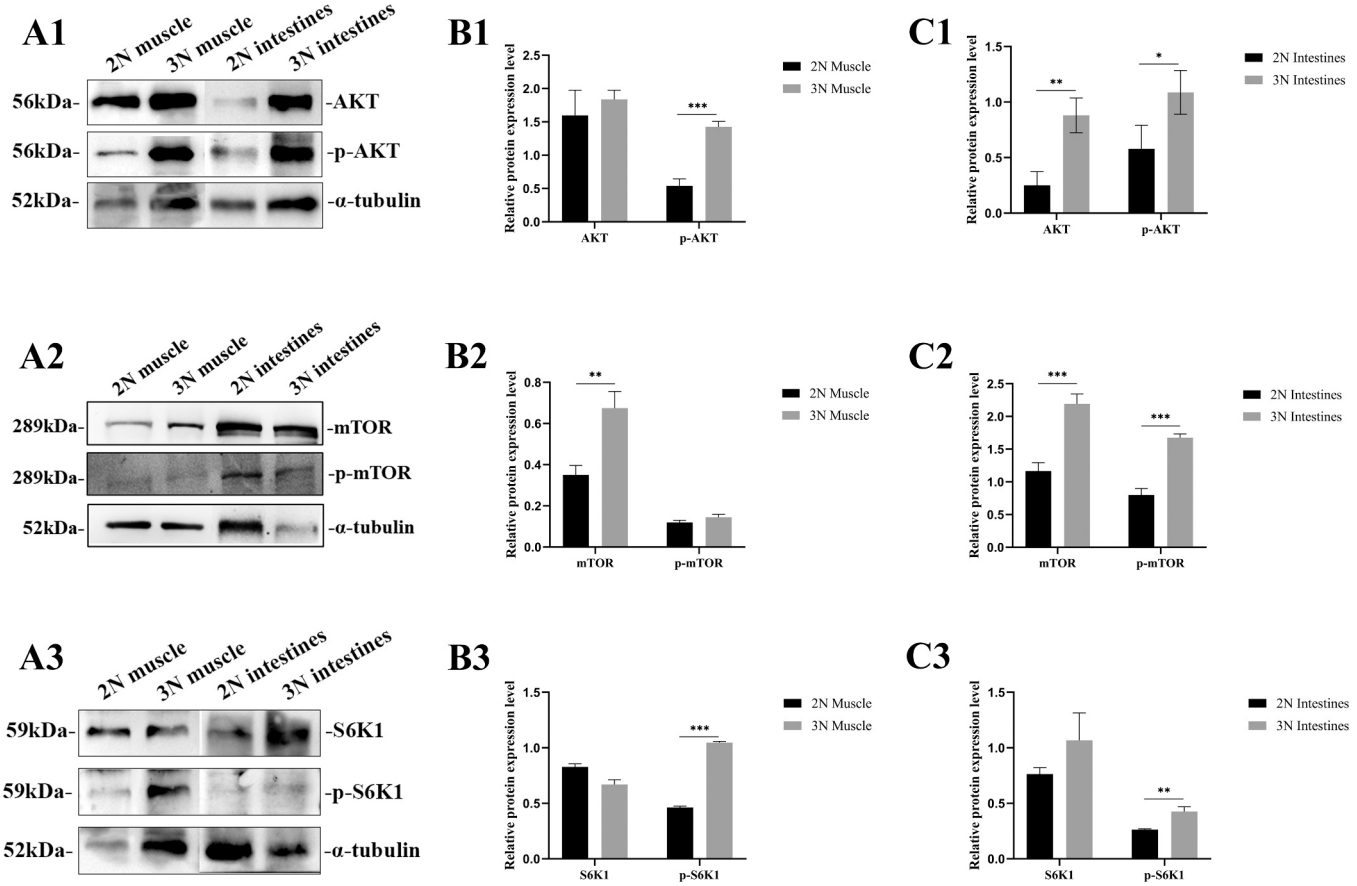

Fig. 2. Protein expression levels of mTOR signaling pathway-related genes in muscle and intestinal tissues of red crucian carp and triploid crucian carp. "2N" represents red crucian carp; "3N" represents triploid crucian carp; * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$.

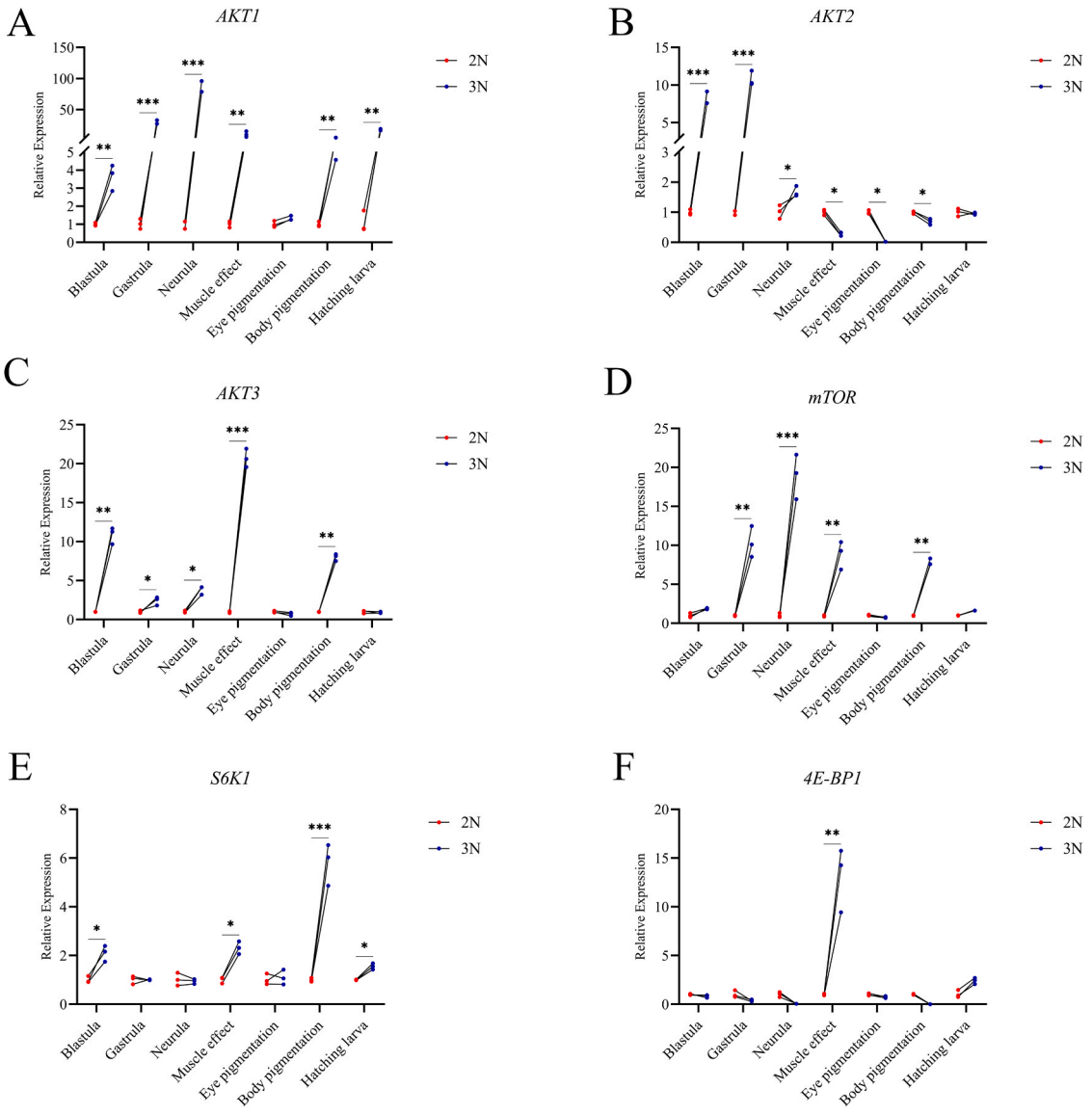

Fig. 3. Expression analysis of mTOR signaling pathway related genes in the embryos of red crucian carp and triploid crucian carp at mRNA level. "2N" represents red crucian carp; "3N" represents triploid crucian carp; * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$.

Table 2
Effect of rapamycin treatment on embryonic development of red crucian carp and triploid crucian carp.

Sample	Total number of embryos (pcs)	Total number of dead embryos (pcs)	Number of membrane-emerging embryos (pcs)	Malformation embryo number (pcs)	Mortality rate (%)	Malformation rate (%)
2 N Control	680	102	84	6	15%	0.8%
2 N Treat	1486	781	129	129	52.5%	100%
3 N Control	562	61	66	0	10.8%	0.0%
3 N Treat	913	278	75	47	30.4%	62.7%

(Fig. 4A2-G2, A4-G4). In red crucian carp, the difference in blastula stage was not significant (Fig. 4A1 and A2 black arrows). However, when the control group reached the early gastrula stage, the treated group remained at the blastula stage (Fig. 4B1 and B2 black arrows). Similarly, when the control group reached the neurula stage, the treated group was at the late gastrula stage (Fig. 4C1 and C2 black arrows). Furthermore, when the control group reached the cerebral optic vesicle stage, the treated group was still at the neurula stage (Fig. 4D1 and D2 black arrows). The delay in development continued, as the control group reached the muscle effect stage while the treated group was still at the cerebral optic vesicle stage (Fig. 4E1 and E2 black arrows). At the stage of noticeable body pigmentation in the control group, the treated group showed almost no pigmentation (Fig. 4F1 and F2 black arrows). Lastly, when the control group reached hatching larva stage, exhibiting dense pigmentation and fully digested yolk sac, the treated group showed delayed pigmentation and incomplete digestion of the yolk sacs (Fig. 4G1 and G2 black arrows). The same delayed development pattern was observed in triploid crucian carp (Fig. 4A3-G3 white arrows).

Effects of rapamycin treatment on the mRNA expression levels of mTOR signaling pathway-related genes were shown in Fig. 5. For red crucian carp, *mTOR* expression was significantly down-regulated in the treated group at both blastula and hatching larva stages, with no significant difference in expression levels at the gastrula, neurula, muscle effect, and eye pigmentation stages (Fig. 5A), while significant up-regulation was observed at the body pigmentation stage. Compared to control embryos, *S6K1* showed significant down-regulation at the gastrula, neurula, and muscle effect stages, followed by up-regulation at the eye pigmentation and body pigmentation stages (Fig. 5B). Compared to control embryos, *4E-BP1* was significantly down-regulated at the gastrula and eye pigmentation stages, and there were no significant changes at the blastula, neurula, muscle effect and body pigmentation stages (Fig. 5C). For triploid crucian carp, *mTOR* expression did not change much in the treated embryos at blastula and gastrula stages, while it was down-regulated at the three key stages of embryonic development, namely, neurula, muscle effect, and eye pigmentation stages, and then significantly up-regulated at the body pigmentation and hatching larva stages (Fig. 5A). Compared with the control group, *S6K1* showed up-regulation from blastula stage to muscle effect stage, and showed down-regulation from eye pigmentation to hatching larva stage (Fig. 5B). *4E-BP1* showed significant down-regulation in blastula and eye pigmentation stages, and up-regulation in the hatching larva period (Fig. 5C).

3.4. Effect of AKT2 gene overexpression on the growth of zebrafish embryos

In the muscle effect embryos, the expression levels of other two genes (*AKT1, AKT3*) were significantly up-regulated in triploid crucian carp, while the expression of *AKT2* was significantly down-regulated (Fig. 3). To further understand the regulatory effect of *AKT2* gene on the *mTOR* expression in embryos, we conducted *AKT2* overexpression experiments in zebrafish (Fig. S1, Fig. 6A). Observation showed that the growth rate of *AKT2* overexpressed embryos was fast compared to NC group (empty vector for injection) (Fig. 6B). In overexpressed group, eye development has already completed while NC group was still at the cerebral optic vesicle stage at 36 hpf (Fig. 6B red arrows). At 60 hpf, the overexpressed group displayed considerably more pigmentation compared to NC group (Fig. 6B white arrows). At 72 hpf, both groups of embryos had reached to hatching larva stage, the overexpressed embryos exhibited more body pigmentation compared to NC groups (Fig. 6B black arrows), indicating that overexpression of *AKT2* gene promoted the development of zebrafish embryos.

We collected embryos during the muscle effect, pigmentation and hatching larva stages for qRT-PCR (Fig. 6C), and the results indicated that during muscle effect period, the expression of four genes (*AKT2*, *mTOR*, *S6K1*, and *4E-BP1*) was higher in overexpressed embryos

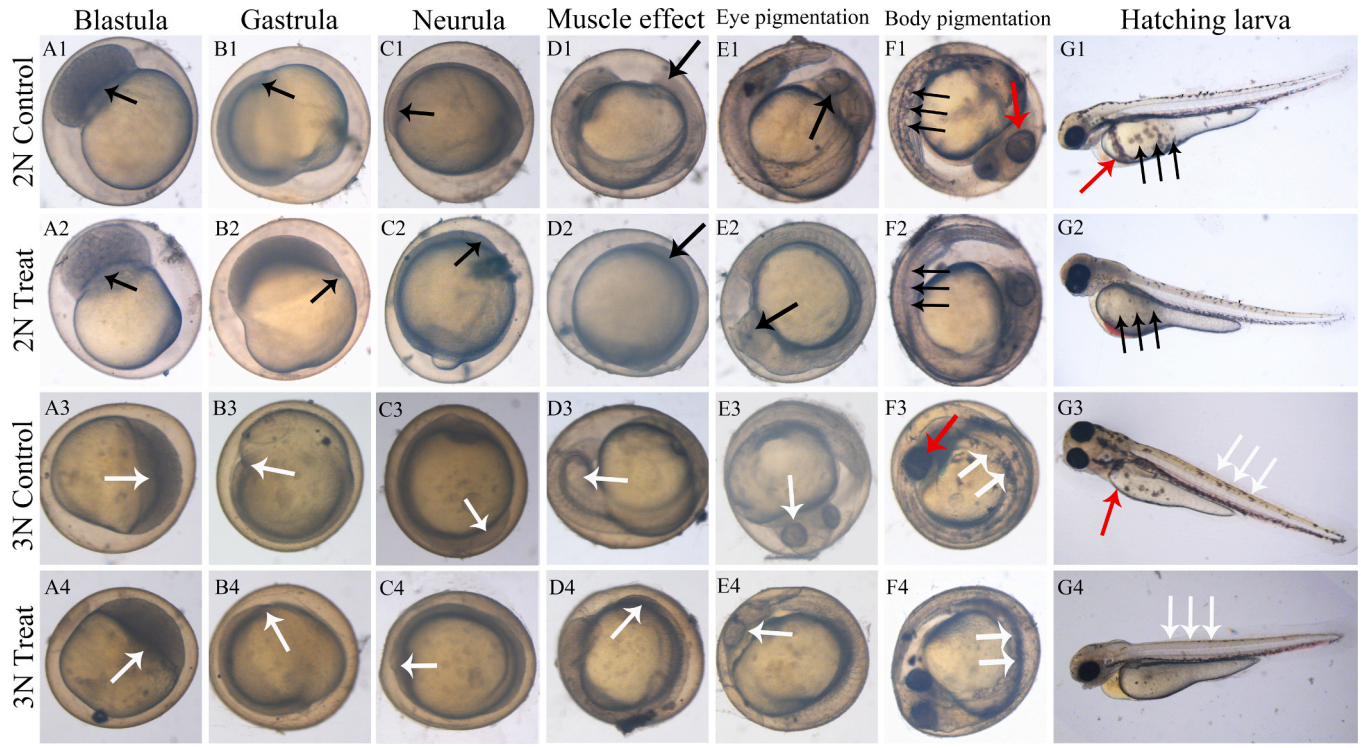

Fig. 4. Effects of rapamycin treatment on embryonic growth of red crucian carp and triploid crucian carp. "2 N Control" represents DMSO treated red crucian carp embryos; "2 N Treat" represents rapamycin treated red crucian carp embryos; "3 N Control" represents DMSO treated triploid crucian carp embryos; "3 N Treat" represents rapamycin treated triploid crucian carp embryos.

compared to NC embryos. Similarly, during pigmentation period, the expression of *AKT2, mTOR*, and *4E-BP1* was higher in the overexpressed embryos compared to NC group, while the expression of *S6K1* was lower than that of NC embryos, and all the differences were significant except for *mTOR*. Finally, the expression of *AKT2*, *mTOR*, *4E-BP1*, and *S6K1* was all higher in the overexpressed embryos compared to NC group during the hatching larva period.

4. Discussion

In this study, the expression of mTOR signaling pathway-related genes (*AKTs*, *mTOR*, and *S6K1*) were higher in the muscle and intestine tissues of triploid crucian carp than those of red crucian carp, not only in their mRNA levels, but also in protein and phosphorylation levels. Moreover, throughout the embryonic development process, triploid crucian carp exhibited a faster growth rate and the higher mRNA levels of *mTOR* gene compared to red crucian carp, especially in the neurula and muscle effect stages. Rapamycin could decrease of *mTOR* activity and induce the developmental retardation of embryos in red crucian carp and triploid crucian carp. Our results confirmed that the mTOR signaling pathway was involved in the embryonic development and rapid growth of triploid crucian carp.

The rapid growth of crucian carp was positively correlated with protein synthesis (Chen et al., 2009), and stimulation of mTOR signaling pathway could enhance protein synthesis in fish (Wu et al., 2021). We noted that the eliminated expression of *mTOR* by rapamycin was observed in the stages of neurula, muscle effect, and eye pigmentation, but significantly up-regulated at the body pigmentation and hatching larva stages. As the embryo developed, the inhibitory efficiency intensified, leading to noticeable downregulation during the neurula and muscle effect phases. However, the developing embryonic organism itself had a mechanism to adapt and balance against the inhibitor rapamycin, preventing a continuous decline in *mTOR* activity. Consequently, as the embryo developed to a certain stage, the activity of *mTOR* gradually recovered. This is why the expected changes were found in the stages of eye pigmentation, body pigmentation, and hatching larva. Meanwhile, during embryonic development, *S6K1* was more highly expressed in triploid crucian carp embryos, in contrast, *4E-BP1* was down-regulated in triploid crucian carp embryos at most embryonic developmental stages. After rapamycin treatment, *S6K1* and *4E-BP1* had opposite expression trends to *mTOR* during the muscle effect period, suggesting that *S6K1* and *4E-BP1* may be inversely regulated by *mTOR* during embryonic development. These results that were inconsistent with expectations required further analysis.

It was reported that mTOR signaling pathway was regulated by upstream *AKTs*, such as *AKT1*, *AKT2* and *AKT3* (Brazil and Hemmings, 2001). Our results indicated that *AKT1, AKT2, and AKT3* were all higher expression in the muscle and intestinal tissues of triploid crucian carp than those of red crucian carp (Fig. 1 and 2). But the expression of *AKT1* and *AKT3* genes (except eye pigmentation and hatching larva stages) was higher in triploid crucian carp embryos compared to red crucian carp embryos, while the expression of *AKT2* gene was significantly downregulated in triploid crucian carp after the neurula stage. Conversely, overexpression of *AKT2* could promote the development of zebrafish embryos, and its downstream genes, such as *mTOR, S6K1,* and *4E-BP1* were significantly up-regulated at the embryos of muscle effect stage and hatching larva. *AKT2* was reported to play a role in regulating insulin homeostasis and glucose metabolism (Cho et al., 2001; Tan et al., 2007). Zhang et al. generated zebrafish lacking *AKT2* gene via CRISPR/Cas9 technology and also found that growth retardation was exhibited in *AKT2*-null zebrafish (Zhang et al., 2017). However, further investigated is required to elucidate the specific molecular mechanisms of *AKT2* in the rapid growth of fish.

In conclusion, mTOR signaling pathway played a crucial role in the regulation of embryonic development and growth in triploid crucian carp and red crucian carp. This research provided further evidence for

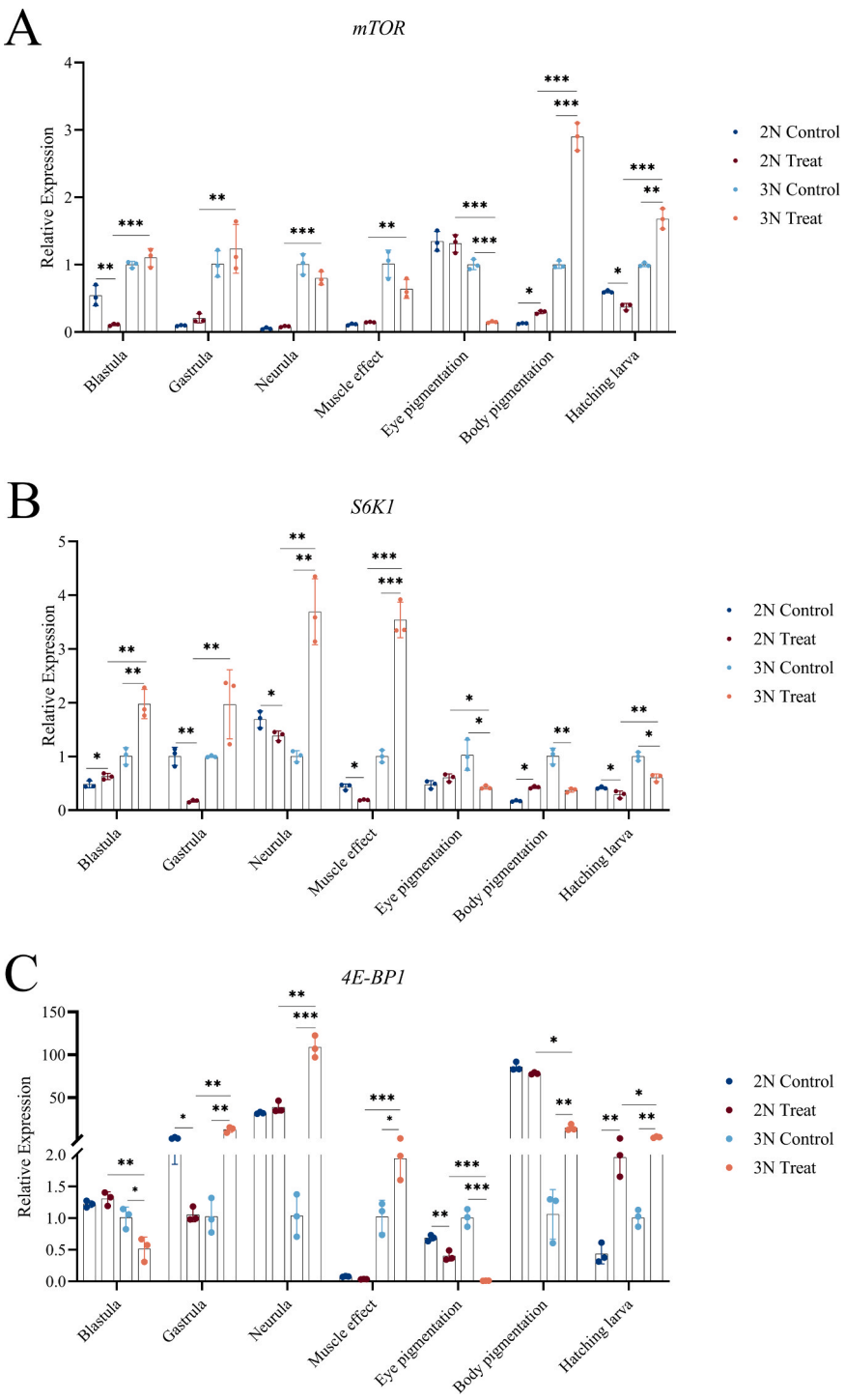

Fig. 5. Expression analysis of mTOR signaling pathway related genes at mRNA levels in red crucian carp and triploid crucian carp embryos under rapamycin treatment. "2N Control" represents DMSO treated red crucian carp embryos; "2N Treat" represents rapamycin treated red crucian carp embryos; "3N Control" represents DMSO treated triploid crucian carp embryos; "3N Treat" represents rapamycin treated triploid crucian carp embryos; * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$, **** represents $P < 0.0001$.

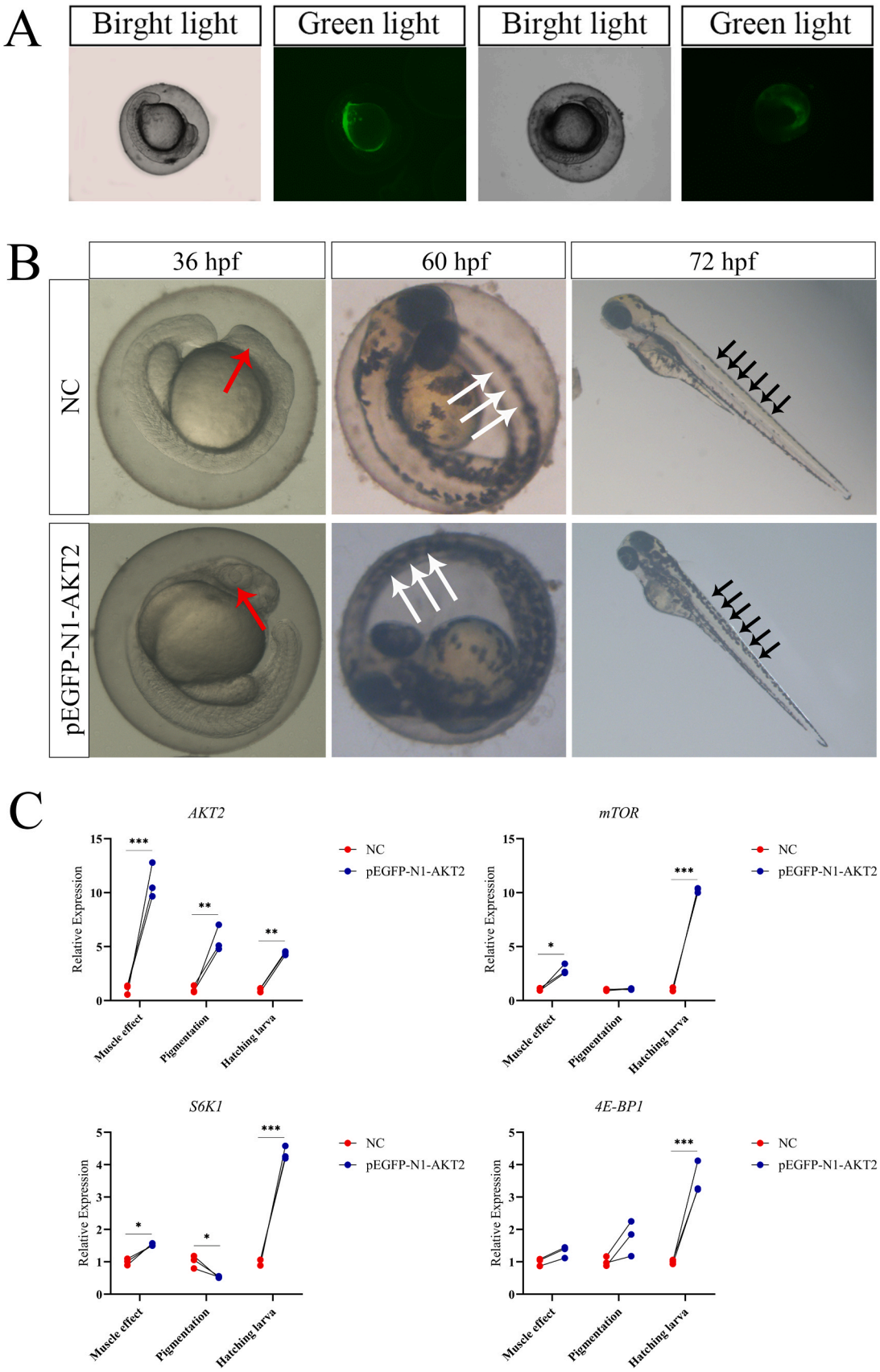

Fig. 6. Fluorescence screening of zebrafish embryos after microinjection and changes in their developmental rate. (A) Photographic results of fluorescence screening of zebrafish after microinjection; (B) Phenotypic changes in developmental speed of zebrafish embryos after microinjection, red arrows: differences in eye development at 36 hpf, white arrows: changes in body color at 60 hpf; black arrows: deposition of body pigment at 72 hpf; (C) Differences in mRNA levels between NC (empty vector for injection) and pEGFP-N1-AKT2 groups during muscle effect, pigmentation and hatching larva stages.

the involvement of the mTOR signaling pathway in the growth and embryonic development of fish, opening up new avenues for exploring fish growth regulation mechanism.

CRediT authorship contribution statement

Yamei Xiao, Wenbin Liu, Zhen Huang and **Liuye Dai**: Design the experiments and organize and write the manuscript. **Wenbin Liu, Zhen Huang, Liuye Dai, Fangyuan Peng, Lingwei Tang, Xuejing Wang, Jiayan Chen, Jinhui Liu, Wen Fu** and **Liangyue Peng**: Carry out the experiments. **Zhen Huang and Liuye Dai**: conduct the statistical analysis and write the discussion.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2023.101860.

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