

Research Paper

C1q-like Factor, a Target of miR-430, Regulates Primordial Germ Cell Development in Early Embryos of *Carassius auratus*

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Abstract

C1q-like is a significant maternal factor of TNF/C1q super-family, and the abundant protein has been observed in both mature eggs of *Carassius auratus* and *Carassius auratus gibelio*, but its biological function in early embryo development has remained unclear. In this study, we firstly revealed a high level of maternal C1q-like transcript existence only in mature eggs of *Carassius auratus*, whereas no any maternal C1q-like transcript was observed in that of *Carassius auratus gibelio*. During embryonic development, the C1q-like zygotic expression begins around cardiopalms stage in embryos of both *Carassius auratus* and *Carassius auratus gibelio*. Then, we examined the biological role of C1q-like by morpholino-mediated knockdown in early embryo development. Knockdown of *CaOC1q* resulted in a significant reduction of primordial germ cells (PGCs) in *Carassius auratus*, as shown by whole mount *in situ* hybridization with *vasa*-specific RNA probe, fluorescence immunostaining of *vasa* protein, and GFP imaging of the GFP-nanos1-3'UTR mRNA reporter. *In vitro* and *in vivo* evidence indicated that a microRNA, miR-430 could repress the C1q-like expression and PGC development. These data suggest that C1q-like should be a direct target of miR-430 and play an essential role in PGC development of *Carassius auratus*.

Key words: C1q-like; microRNA; miR-430; knockdown; primordial germ cell; early embryogenesis

Introduction

Primordial germ cells (PGCs), the founder cells of both female and male gametes, initially appear outside the gonadal region, and then are specified and migrated to the genital ridges [1-3]. The first discovered molecular marker for PGCs in fish is *vasa* [4, 5], while several other types of factors, such as Kit ligand, FGFs, leukemia inhibitory factor (LIF) and interleukin-4 (IL4) [6-9], have been shown to stimulate PGCs proliferation and/or survival *in vitro*. However, it is not clear what roles these factors have in regulating PGC development *in vivo*. Previously, Kawase et al. observed that addition of TNF- α to culture medium stimulated proliferation of PGCs without transforming them into embryonic stem cells, and that its effect

was specific for the PGCs at younger stages before and during their migration to gonads [10]. C1q-domain-containing proteins have been shown to be members of TNF/C1q super-family, and to possess diverse functions in immune response, cell activity and organ development [11-14], but their roles in PGC development have not been observed so far.

Gibel carp (*Carassius auratus gibelio* Bloch), one of the most important freshwater aquaculture species in China, has been extensively studied for its dual modes of unisexual and sexual reproduction [15-17]. Many key maternal factors, such as *Vasa*, *Dazl*, *H2Afl0* and *Spindlin*, have been found to play important roles in early embryonic development [18-23].

Significantly, two homologous ovary-specific *C1q-like* genes, *CaOC1q* (*Carassius auratus* ovary-specific *C1q-like*) and *CagOC1q* (*Carassius auratus gibelio* ovary-specific *C1q-like*), have been identified from gonochoristic *Carassius auratus* and gynogenetic *Carassius auratus gibelio* [11], respectively. They are highly conserved, and there are abundant maternal proteins to be associated with cortical granules in mature eggs [13]. As an essential maternal factor, however, its physiological role in early embryonic development is still unknown.

MicroRNAs (miRNAs) are a class of 22~24-nucleotide RNAs which usually bind to 3'UTR of target genes, and lead to target mRNA degradation or translation inhibition [24,25]. MiR-430, a well-characterized miRNA expressed at maternal-zygotic transition (MZT) stage, has been shown to target a large number of maternally expressed mRNAs and to promote their deadenylation and degradation [26, 27]. During zebrafish embryonic development, miR-430 regulates PGC development through controlling *sdf1a*, *cxc7*, *TDRD7* and *nanos1* expression [28-30]. In this study, we found a significant difference of *C1q-like* transcriptional level in mature eggs between *Carassius auratus* and *Carassius auratus gibelio*, in which there was abundant maternal *C1q-like* protein in both mature eggs, but a high level of maternal *C1q* transcript existed only in mature eggs of *Carassius auratus*, whereas almost no any maternal transcript was observed in mature eggs of *Carassius auratus gibelio*. The finding provided a good chance to use morpholino-mediated knockdown in *Carassius auratus* to study physiological role of the *C1q-like* in early embryonic development. Thereby, we show that the *C1q-like* factor, which is a target of miR-430, regulates PGC development in early embryos of *Carassius auratus*.

Materials and methods

Materials

Carassius auratus gibelio and *Carassius auratus* were obtained from Guanqiao Experimental Station, Institute of Hydrobiology, Chinese Academy of Sciences. During reproduction season, the mature fish was selected and artificially induced into spawning by two intraperitoneal injections with a mixture of acetone-dried carp pituitary, HCG and LRH-A as described previously [13,22]. Experimental protocols used here were approved by the animal care and use committee of Institute of Hydrobiology, Chinese Academy of Sciences.

Virtual Northern Blot and RT-PCR analysis

Differential transcription of oocyte-specific *C1q-like* was analyzed by virtual Northern blotting as

described previously [31]. Briefly, 1µg PCR product amplified by forward primer *C1q-like-F* (5'-GAGCTTGAGAACTGAATGAC-3') and downstream primer *C1q-like-R* (5'-TGACTGCCATTGTTGTTCCAC-3') was labeled as the probe using DIG-High Primer system (Boehringer Mannheim). The α -tubulin control amplified by forward primer (5'-GTGCACTGGTCTTCAGGGGTT-3') and reverse primer (5'-GGGAAGTGGATGCGTGGGTAT-3') was done as the loading control. Semi-quantitative RT-PCR was carried out on 20 eggs and embryos at different stages of oogenesis and embryonic development as previously described [32]. The expression change of *C1q-like* regulated by miR-430 and miR-430 expression during embryo development was measured by quantitative RT-PCR as previously described [33]. *a-tubulin* was used as the internal control for RT-PCR. All the experiments were repeated at least three times.

Whole mount RNA in situ hybridization and antibody in situ staining

The antisense DIG-labeled RNA probes used in our experiments were generated from *CaOC1q*, *CagOC1q* and *vasa* mRNA sequences, and whole-mount RNA *in situ* hybridization was performed as described [34,35]. All the procedures were RNase-free until the hybridization finished. Finally, embryos were stained with BCIP/NBT and visualized with a microscope. The pictures were taken with a Leica camera.

Antibody immunofluorescence labeling was done as previously described using *vasa* specific antibody [18]. Finally, embryos were incubated with secondary FITC-labeled goat-anti-rabbit antibody in dark for 2h at room temperature and visualized with a Leica fluorescence microscope.

Morpholino microinjection

C1q-like morpholino (*C1q-like* MO) targeting the translation start codon of *CaOC1q* and control morpholino (Cont MO) were synthesized by Gene Tools, and subsequently injected into animal pole of fertilized eggs at the one-cell stage (10 ng per embryo, an optimal concentration without causing non-specific defects) as described previously [36]. The morpholino sequences were as follows: *C1q-like* MO, 5'-GCAGTACCATTTGTAAGACCACCAT-3'; Control MO (Cont MO), 5'-CCTCTTACCTCAGTTACAA TTTATA-3'. MiR-430 mimic and control were purchased from Genepharma (Shanghai, China), and about 0.6 ng was injected in each embryo. After injection, embryos were incubated at 24.5°C in Holtfreter's medium, which was replaced by oxygen-enriched water one day later.

The full coding sequence of *C1q-like* was amplified and subcloned into pEGFP-N3 (provided by Dr. Qiya Zhang, Institute of Hydrobiology, Chinese Academy of Science) and pCS₂⁺ vector (supplied by Dr. Shuo Lin, University of California, Los Angeles) to generate mammalian expression constructs. For rescue experiment, *C1q-like* ORF with 5-mismatch in the morpholino binding site (no change to the coding protein) was subcloned into the pCS₂⁺ vector. The constructed vector was used for *in vitro* mRNA transcription to generate 5-mis rescue mRNA that could not bind to the morpholino. *GFP-nanos1-3'UTR* reporter was provided by Professor Erez Raz (Institute of Cell Biology, ZMBE, Germany). Capped RNAs were synthesized by SP6 RNA polymerase (Roche) with linearized plasmids, following the manufacturer's instructions. Then the RNAs were re-suspended in water and injected at a concentration of 100 ng/ μ L. The embryos of different development stages were detected by inverted fluorescence microscope (Leica) and photographed by CCD as described previously [37].

Luciferase reporter assay

The 3' untranslated region (3'UTR) of *CaOC1q*, which contains one putative miR-430 binding site, was amplified by PCR and inserted into the *SacI* and *XhoI* sites of the pmir-GLO vector (Promega). The binding site (GTACTT) for miR-430 within the 3'UTR of the *CaOC1q* gene was replaced with GCTAGC to generate a mutant control for the luciferase reporter assays. Grass carp (*Ctenopharyngodon idellus*) ovary cells (abbreviated as CO cells) were transfected with plasmids and microRNA mimics using Lipofectamine 2000 (Invitrogen) in 48-well plates. 48h post transfection, the cells were lysed and assayed for dual reporter (*Firefly* and *Renilla*) activity using a Du-

al-Luciferase reporter assay system (Promega) as described [38,39]. Relative reporter activities were determined by normalizing *Firefly* activity to *Renilla* activity.

Statistical analysis

Data was shown as mean \pm SD. For statistical analysis, non-parametric Mann-Whitney U tests and ANOVA tests were performed with SPSS software (SPSS Inc.). A probability (P) of <0.05 was considered statistically significant.

Results

Significant difference of maternal *C1q-like* transcript in mature eggs between *Carassius auratus* and *Carassius auratus gibelio*

Previous studies have observed abundant maternal *C1q-like* protein existence during oocyte maturation and early embryo development of both *Carassius auratus* and *Carassius auratus gibelio* [11,13]. To further elucidate their transcription pattern and corresponding difference, we performed RT-PCR and virtue Northern blot analyses. As shown in Fig. 1A, high level transcription of *CagOC1q* and *CaOC1q* occurs during oogenesis and oocyte maturation from phase I to phase IV, but a significant expression difference is observed from mature eggs between *Carassius auratus* and *Carassius auratus gibelio*, in which a high level of maternal *C1q* transcript exists only in mature eggs of *Carassius auratus*, whereas almost no any maternal *C1q* transcript is detected in mature eggs of *Carassius auratus gibelio*. Virtue Northern Blot further showed a 1.2kb band in *Carassius auratus* mature eggs which was consistent with the molecular weight of the full length sequence of *CaOC1q* cDNA, while no any corresponding signal was appeared in *Carassius auratus gibelio* mature eggs

except some non-specific signals (Fig.1B). These data indicate that a high level of maternal *C1q-like* transcript exists in mature eggs of *Carassius auratus*, whereas there is no any maternal *C1q-like* transcript in that of *Carassius auratus gibelio*.

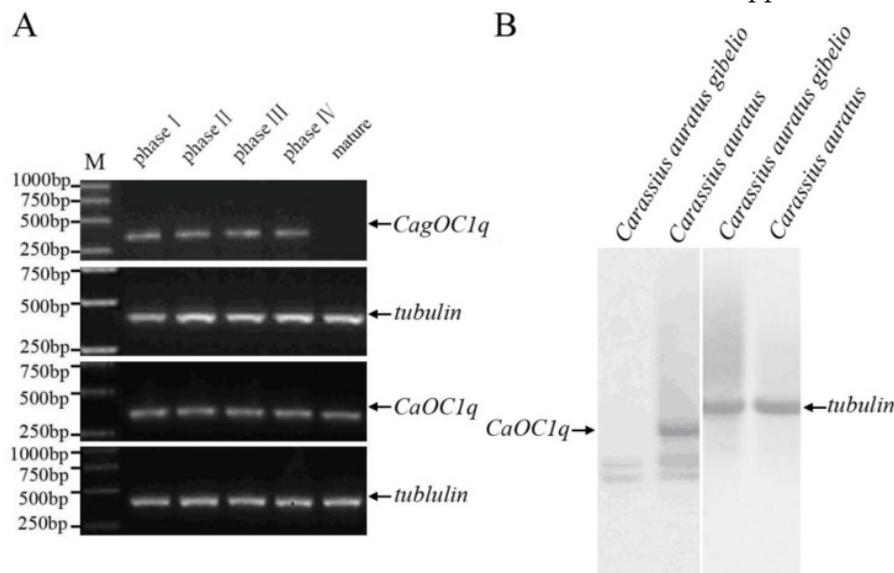


Fig.1. Significant difference of maternal *C1q-like* transcript in mature eggs between *Carassius auratus* and *Carassius auratus gibelio*. (A) RT-PCR detection of *C1q-like* during oogenesis and oocyte maturation from phase I to phase IV oocytes and in mature eggs. (B) Virtue Northern Blot detection of *CagOC1q* and *CaOC1q* in mature eggs.

Zygotic expression pattern of C1q-like during embryonic development in *Carassius auratus* and *Carassius auratus gibelio*

We performed RT-PCR analysis to determine the expression pattern of *C1q-like* during embryo development. As shown in Fig. 2A, the zygotic expression of *C1q-like* occurs around cardiopalmus stage during embryonic development in both *Carassius auratus* and *Carassius auratus gibelio*, but the maternal *C1q-like* transcript exists only in early embryos of *Carassius auratus*, and holds to cardiopalmus stage until the zygotic expression has begun. However, no any maternal *C1q-like* transcript is observed in early embryos of *Carassius auratus gibelio* before the zygotic expres-

sion. Whole mount *in situ* hybridization was also used to investigate the spatial and temporal pattern difference of ovary-specific *C1q-like* in early embryos between *Carassius auratus* and *Carassius auratus gibelio*. As shown in Fig. 2B, no any positive signal is observed at the corresponding region of *Carassius auratus gibelio* embryos at that stage (Fig.2B-a, b), whereas positive *CaOC1q* transcript signal is detected in the corresponding ventral genital ridge region of the embryo trunk of *Carassius auratus* at muscle affection stage (Fig.2B-c, d). The data implicate that the maternal ovary-specific *C1q-like* transcript might be localized into genital ridge region before the zygote *CaOC1q* expression.

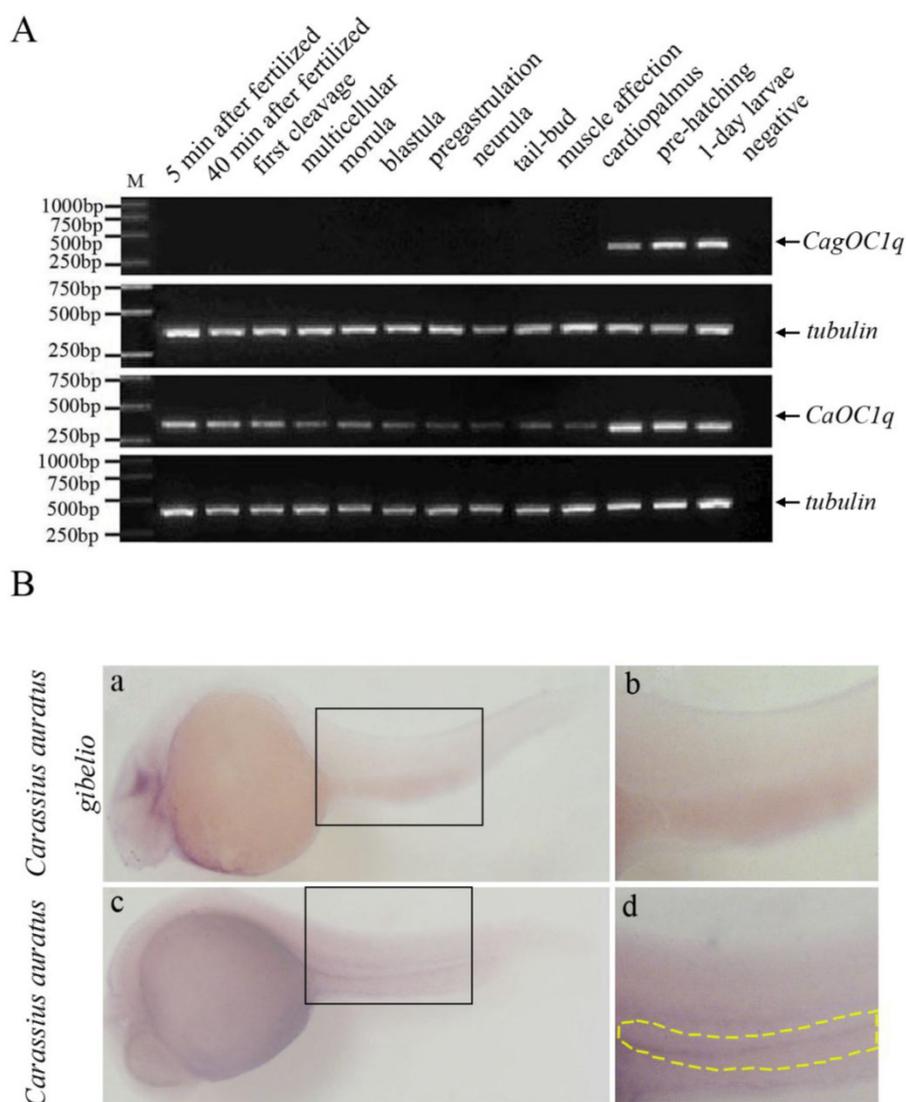
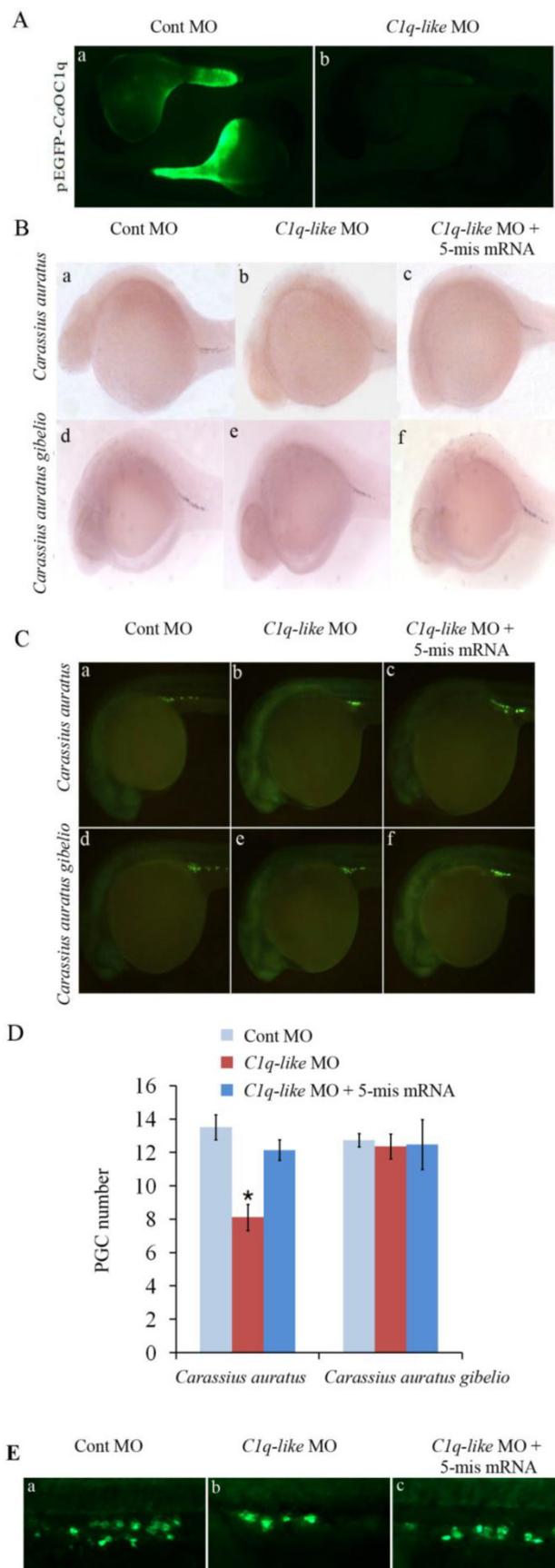


Fig.2. (A) RT-PCR detection of *C1q-like* expression during embryonic development in *Carassius auratus gibelio* and *Carassius auratus*. (B) Whole mount RNA *in situ* hybridization of *Carassius auratus gibelio* (a) and *Carassius auratus* (c) embryos at muscle affection stage. Panels b and d show the corresponding amplification signals of genital ridge in the selected region of panels a and c. Embryos are shown laterally with anterior to the left.

C1q-like knockdown impairs PGC development in *Carassius auratus*

The existence of maternal *C1q-like* transcript in early embryos of *Carassius auratus* gave us a good chance to use morpholino-mediated knockdown to study functional role of the *C1q-like* in early embryonic development of fish. Firstly, we checked the efficacy of the designed morpholino by co-injecting morpholino with a *CaOC1q* expression construct fused with green fluorescent protein (GFP) into the fertilized eggs of *Carassius auratus*. *C1q-like* MO specifically reduced expression of GFP at cardiopalmus stage in 100% of embryos (n=100) (Fig. 3A). In comparison with strong GFP signal in the control group (Fig. 3A-a), there was no detectable signal when co-injected with the *C1q-like* morpholino (n=100) (Fig.3A-b). Finally, the inhibition efficacy of *C1q-like* MO was the same in *Carassius auratus gibelio* (n=100, data not shown). This revealed the ability of the *C1q-like* MO to inhibit protein production from its target sequence. Further, no obvious developmental abnormalities or mortalities were observed in either the *C1q-like* MO or Cont MO injected embryos (n=100, data not shown).

Fig.3. Knockdown of *C1q-like* results in defects of PGCs development in *Carassius auratus*. (A) Validation of knockdown efficiency of *C1q-like* morpholino. Embryos were injected with Cont MO (a) or *C1q-like* MO (b) with *C1q-like*-GFP fusion protein expression vector. *Vasa* RNA in situ hybridization (B) and antibody immunostaining (C) to identify PGCs in cardiopalmus stage embryos. (a, d) Cont MO injected embryos; (b, e) *C1q-like* MO injected embryos; (c, f) *C1q-like* MO + 5-mis mRNA injected embryos. (D) Quantification of *vasa*-positive PGC number in the Cont MO, *C1q-like* MO and *C1q-like* MO + 5-mis mRNA injected embryos. (E) Identification of PGCs by GFP-nanos1-3'UTR reporter in *Carassius auratus*.



In order to analyze the function of *C1q-like* on PGC development during early embryogenesis, we injected *C1q-like* MO into the fertilized eggs of *Carassius auratus* and *Carassius auratus gibelio*, and PGCs were recognized through whole mount *in situ* hybridization with vasa-specific RNA probe (Fig.3B). Consistent with the absence of maternal *C1q-like* transcript in *Carassius auratus gibelio*, knockdown of *CaOC1q* didn't obviously change the number of PGCs (Fig.3B-d, e, f). In contrast, *CaOC1q* knockdown significantly reduced vasa-labeled PGCs in *Carassius auratus* (Fig.3B-b). Moreover, the reduction of PGC signals could be recovered by 5-mis *CaOC1q* mRNA (Fig.3B-c). In order to further determine the number of PGCs, they were identified and quantified by fluorescence immunostaining of vasa protein (Fig.3C). Similar to the result in Fig.3B, knockdown of *C1q-like* didn't obviously change the number of PGCs in *Carassius auratus gibelio* (Fig.3C-d, e, f and D). However, the average number of PGCs in the *C1q-like* MO injected embryos in *Carassius auratus* (Fig.3C-b and D) was 39.8% lower than that of the Cont MO injected embryos (Fig.3C-a and D). The specific effect of *C1q-like* MO on PGCs was confirmed through analyzing rescue of PGCs by overexpression of 5-mis *CaOC1q* mRNA (Fig.3C-c and D). To further address this, we injected GFP-nanos1-3'UTR mRNA with morpholino into fertilized embryo, and then imaged the fluorescence of GFP. The GFP-nanos1-3'UTR reporter further revealed that *C1q-like* plays an important role in PGC development in *Carassius auratus* (Fig.3E).

miR-430 regulates PGCs development in *Carassius auratus*

One miR-430 precursor was found from ovary transcriptome of *Carassius auratus gibelio* sequenced by 454 GS-FLX platform, and its sequence was found to be identical to that in *Carassius auratus* (data not shown). Compared with *Cyprinus carpio* and *Danio rerio*, the miR-430 precursor in *Carassius auratus* has very high identity to miR-430b, and the mature microRNA sequence was consistent with that in *Cyprinus carpio* (Fig. 4A). Moreover, the miR-430 precursor in *Carassius auratus* showed typical stem-loop structure (Fig. 4B) and RT-PCR analysis detected its expression in embryos at tail-bud stage and muscle affection stage (Fig. 4C).

Furthermore, we observed one binding site for miR-430b in *C1q-like* 3'UTR, which contains only one mismatch within the seed-matched site (Fig. 5A). In teleost fish, miR-430 is essential for primordial germ cell development by targeting *sdf1a*, *cxcr7*, *TDRD7* and *nanos1* mRNA [28-30]. Although miR-430 was widely studied in zebrafish development, its function was unknown in *Carassius auratus*. Therefore, we evaluated the effect of miR-430 over-expression on PGCs development in *Carassius auratus*. Ectopic expression of miR-430 apparently reduced vasa-labeled PGCs in *Carassius auratus* compared with the control miRNA mimic, as shown by vasa antibody *in situ* staining (Fig.5B). The reduced level was above 50% indicated by the quantified data (Fig.5C).

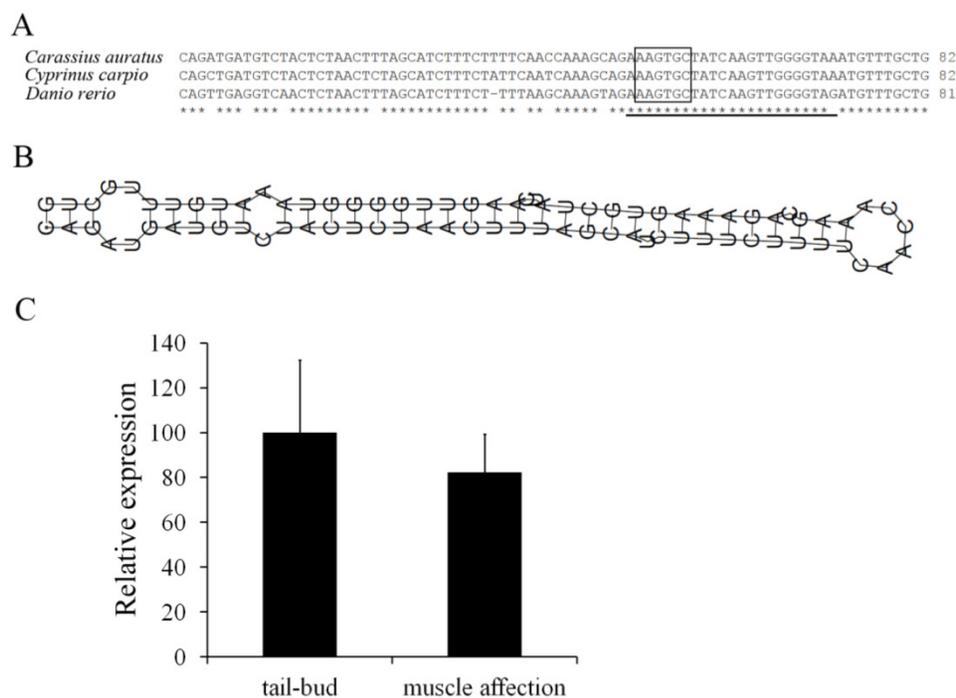


Fig.4. Molecular characterization of miR-430 and its embryonic expression in *Carassius auratus*. (A) Multiple alignments of *Carassius auratus*, *Cyprinus carpio* and *Danio rerio* miR-430 precursors. Boxed letters represent seed sequence of miR-430 and the lined letters represent miR-430 mature sequence. (B) Stem-loop structure of miR-430 precursor in *Carassius auratus*. (C) Real-time PCR detection of miR-430 expression in embryos at tail-bud stage and muscle affection stage.

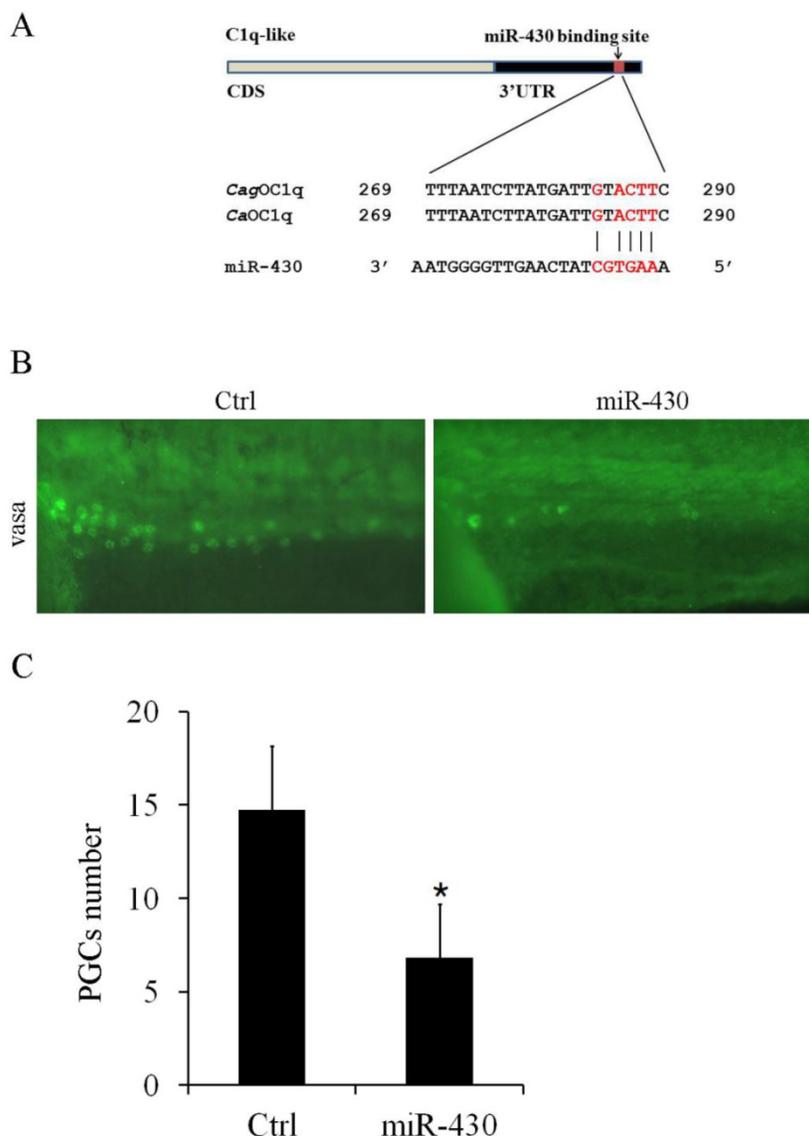


Fig.5. miR-430 regulates PGCs development in *Carassius auratus*. (A) Sequence alignment of C1q-like 3'UTR and miR-430. Red color letters represent seed sequence of miR-430 and its binding position. (B) Representative images of PGCs development in cardiopalms stage embryos, as shown by vasa antibody immunostaining. (C) Quantification of PGCs numbers in the embryos injected with miR-430 mimic and its control.

CaOC1q is an important target of miR-430

To determine the regulation of *CaOC1q* by miR-430, we firstly performed a luciferase reporter assay by linking the 3'UTR of *CaOC1q* to the C-terminus of *Firefly* luciferase present in pmirGLO vector. PmirGLO/3'-UTR and its mutant vectors (as described in methods) were co-transfected with miR-430 or control microRNA mimic into CO cells. Compared to control microRNA-mimics, luciferase activity was significantly reduced (up to 38%) when the PmirGLO/3'-UTR reporter was co-transfected with miR-430. In contrast, miR-430 did not change the activity of a luciferase reporter when the binding site for miR-430 in 3'UTR of *CaOC1q* was mutated (Fig.

6A). Furthermore, *in vivo* study showed that over-expression of miR-430 resulted in a significant decrease of *CaOC1q* expression compared with the control miRNA mimic in 30 hpf embryos (Fig. 6B). However, no difference in expression levels of *CaOC1q* were detected in 30 hpf embryos when *CaOC1q* express construct without its specific 3'UTR was co-injected with the control and miR-430 mimic (Fig. 6C). In addition, we injected pmirGLO/ *CaOC1q* 3' -UTR reporter at 1-cell stage, and found that *CaOC1q* mRNA expression was increased (Fig. 6D), for the reporter could bind to endogenous miR-430 and block *CaOC1q* degradation. Hence, the regulation of *CaOC1q* expression is specific to miR-430.

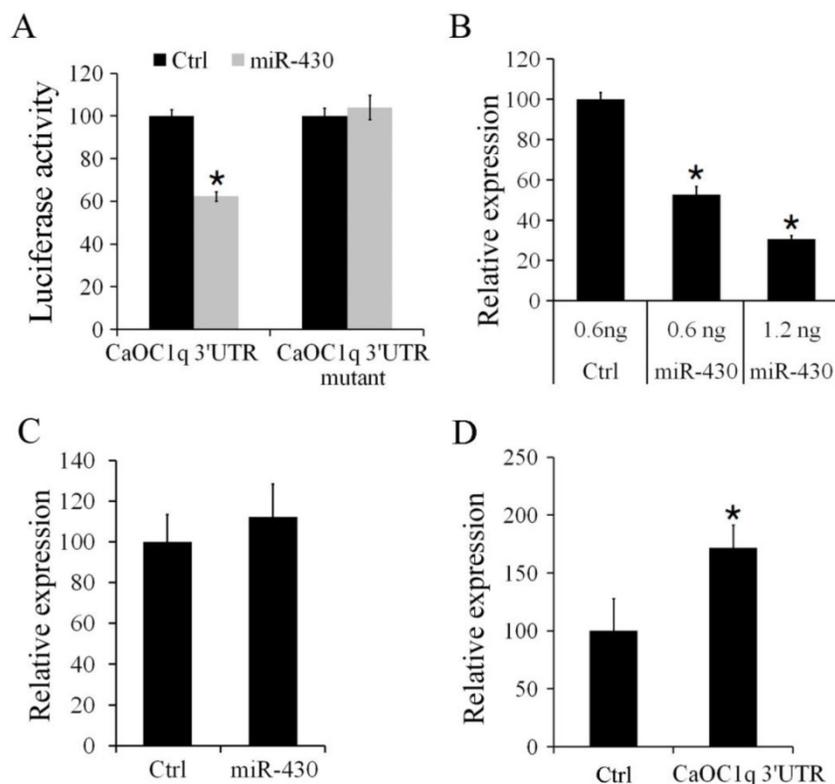


Fig.6. *CaOC1q* is a direct target of miR-430. (A) miR-430 suppressed the activity of pmirGLO reporter that is linked to the 3'UTR of *CaOC1q*, but not mutant 3'UTR of *CaOC1q*. The *Firefly* activity was normalized to *Renilla* expression, which was used as a control for transfection efficiency. (B) Over-expression of miR-430 reduced endogenous *CaOC1q* mRNA transcripts, as determined by qRT-PCR. (C) miR-430 had no effect on ectopic *CaOC1q* expression when embryos were injected with *CaOC1q* express construct without its specific 3'UTR. (D) *CaOC1q* mRNA expression was increased when embryos were injected with reporter which ectopically expressed *CaOC1q* 3'UTR.

Discussion

In recent years, TNF family members have been reported to be involved in the process of oogenesis and embryogenesis, including primordial germ cell development [10, 40, 41]. As an important member of TNF/C1q super-family reported in fishes, *C1q-like* has been studied in oogenesis and oocyte maturation in *Carassius auratus* and *Carassius auratus gibelio* [11, 13]. In this study, we revealed the different expression pattern of *C1q-like* between *Carassius auratus* and *Carassius auratus gibelio*. Furthermore, we have identified and characterized *C1q-like* as a direct target of miR-430, and investigated its function on PGC development through the morpholino-mediated knockdown.

In this study, we have observed that *C1q-like* has basically consistent transcription pattern from stage I oocytes to stage IV oocytes in both species, but a high level of *CaOC1q* transcript exists only in mature eggs of *Carassius auratus*, whereas there is no any maternal *CaOC1q* mRNA in mature eggs of *Carassius auratus gibelio* (Fig.1). The observation implicates that maternal *CaOC1q* mRNA may be predominantly expressed by follicle cells and gone along with the follicular cell degeneration and deletion [13]. However,

CaOC1q maternal mRNA is expressed by oocytes and localized in the cytoplasm, and kept its high transcription level during follicular cell apoptosis [11]. RT-PCR analysis indicated that the zygotic transcription started around cardiopalmus (Fig. 2). In addition, knockdown of *CaOC1q* has no effect on PGC development of early embryo (Fig. 3). These findings suggest that maternal *CaOC1q* protein should be sufficient for normal early embryo development in *Carassius auratus gibelio*. Previously, many maternal factors to control early embryo development had been identified by maternal-effect mutant screening in zebrafish [42]. And, some maternal factors had been demonstrated to be required for zebrafish PGCs formation [43, 44]. A unique finding of the current study was that the maternal *CaOC1q* mRNA might be involved in PGC development, because the knockdown of *CaOC1q* resulted in significant decrease of PGC number, implicating that the maternal *CaOC1q* mRNA translation might be necessary for maintaining sufficient level of *CaOC1q* protein to keep normal PGC development. Moreover, MiR-430, which was known to control normal PGC development [26-30], was also revealed to directly target *CaOC1q* and guide its expression at an optimal level in the early embryo development of *Carassius auratus*. Furthermore, sev-

eral other maternal factors involved in oogenesis and early embryo development of *Carassius auratus gibelio*, such as Cyclin A2, Dazl and SNRPC [19, 45, 46], were also predicted to be targeted by miR-430 (data not shown). In future, it would be interesting to elucidate the relationship between miR-430 and maternal mRNA in *Carassius auratus gibelio* and *Carassius auratus*.

Recent studies have revealed several signaling pathways that regulate the proliferation, survival and migration of fish PGCs. For example, chemokine CXCL12 was found to regulate directional migration of PGCs through the G-protein-coupled receptor CXCR4 [47, 48]. IGF1Rb was shown to potentially regulate PGC development via cross-talk with chemokine signaling pathways [49]. In our previous work, zebrafish *C1q-like* was also demonstrated to be required for cell survival during embryonic development [14], and the sequence identity between zebrafish *C1q-like* and *CagOC1q* was relatively high (~70%). Our preliminary analysis indicated that *in vitro* over-expression of *CagOC1q* was able to protect FHM (fathead minnow) cells from ACTD induced apoptosis (data not shown). Further research will be undertaken to explore whether *CaOC1q* is essential for the cell survival of PGCs *in vivo*, and how *C1q-like* interacts with other molecular signals to control early embryo development.

Acknowledgements

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Competing Interests

The authors have declared that no competing interest exists.

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