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Full Length Article

Knockdown of Novel lncRNA *TCONS_00028652* in Zebrafish Affects Embryonic Vasculature Development

Chune Zhou, Shuqiang Zhang, Junguo Ma and Xiaoyu Li*
College of Life Science, Henan Normal University, Xinxiang, Henan 453007, P. R. China
*For correspondence: lixiaoyu65@263.net; 041106@htu.edu.cn
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Abstract

Long non-coding RNAs (lncRNA) are increasingly being regard as potential key regulators of biological process, however, little is known about the function of most of them. The involvement of a novel lncRNA (ENSDART000000150571), previously named *TCONS_00028652*, in intersegmental vessel development was investigated in this study. *TCONS_00028652*, having a single exon (568 bp), is located in chromosome 16 at position from 15786804 to 15786237, which was previously identified as an embryonic and adult heart-enriched lncRNA. Bioinformatics analysis and annotation of *TCONS_00028652* was performed using online databases. Its protein-coding potential was assessed using online softwares: the Coding Potential Assessing Tool (CPAT) and the Coding Potential Calculator (CPC). To verify its real existence, a cloned fragment was proliferated by designing primers against 5' and 3' exon-flanking sequence. Subsequently, spatiotemporal expression during zebrafish embryonic development was determined using real-time quantitative PCR (qPCR) and whole mount *in situ* hybridization. We found that lncRNA *TCONS_00028652* was generally expressed throughout early stages of zebrafish embryonic development and predominantly in embryonic brain, tail, and heart. Knockdown of *TCONS_00028652* using morpholino oligonucleotides (MO) resulted in intersegmental vessel defects, suggesting that *TCONS_00028652* is indispensable for embryonic vascular development in zebrafish. Using Tg (*flil:EGFP*) transgenic fish expressing a cardiovascular marker gene, loss of function experiments confirmed that *TCONS_00028652* was involved in embryonic intersegmental vessel development. Our results may lead to valuable understanding of lncRNAs functions in zebrafish embryonic development and molecular mechanisms of embryonic cardiovascular development. © 2021 Friends Science Publishers

Keywords: lncRNA *TCONS_00028652*; Zebrafish; Embryonic development; Spatiotemporal expression; Intersegmental vessel
Abbreviations: qPCR: quantitative real-time polymerase chain reaction; LncRNA: long noncoding RNA; Se: the primary intersegmental vessel; MO: morpholino oligonucleotide; Tg: transgenic; ANOVA: analysis of variance; DIG: digoxigenin; hpf: hours post-fertilization; CRISPR: clustered regularly interspaced short palindromic repeats; EGFP: enhanced green fluorescent protein; ESCs: embryonic stem cells.

Introduction

Recent applications of high-throughput sequencing technology have uncovered that less than 2% of the human genome encodes proteins (Esteller 2011; ENCODE Project Consortium 2012; Kellis *et al.* 2014) and most of the remaining genome that does not encode protein is made up of so called non-coding RNA (Mattick and Makunin 2006; Mercer *et al.* 2009; Adams *et al.* 2017). On the basis of mature transcript size, less than 200 nucleotide can be defined as small RNAs, greater than 200 nucleotide can be named as long ncRNAs (lncRNAs) and another type of ncRNA is housekeeping small RNAs, referred as regulatory RNAs (*e.g.*, rRNA and tRNA). Amongst small RNAs, microRNAs (miRNAs) are the best studied. However, as the largest number of ncRNAs (Chowdhury *et al.* 2013), lncRNAs have attracted recent attention for the diverse gene regulation activity they provide at transcriptional, post-transcriptional, and epigenetic levels (Gutschner and Diederichs 2012). Hitherto, a large number of lncRNAs have been identified in animals, but their functions remain largely undefined (Atkinson *et al.* 2012; Derrien *et al.* 2012; Batista and Chang 2013).

It is necessary for embryonic development and adult survival to have normal cardiovascular system. In zebrafish, the cardiovascular system is developed before other organ systems during embryogenesis, with circulation beginning as early as 24 h post fertilization (hpf) (Fishman and Chien 1997). A functional vascular system is indispensable for supplying nutrients, hormones, immune cells, and oxygen for developing tissues and organs, as well as for eliminating toxic metabolic waste products. Cardiovascular development is a finely regulated process that necessitated abundant of genes (Kathiriya *et al.* 2015).

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Recent studies have indicated that lncRNAs play key roles in cardiovascular development (Rayner and Liu 2016). Several studies revealed that lncRNAs have a key role in cardiovascular development.

Zebrafish has become as popular animal in many area of research for its more rapid development speed, relatively shorter breeding cycle, and easier genetic manipulation (Vesterlund *et al.* 2011). In particular, they are an exceptional model animal for studying vascular development because they not only have transparent embryos and can be manipulated genetically (Bradbury 2004), but also their embryos can survive without blood circulation for approximately 7 days post fertilization. Thus, it is easy to discern vascular mutants. Transgenic zebrafish expressing enhanced green fluorescent protein (EGFP) [*Tg (flk*:EFGP), *Tg(fli1:*EGFP)] throughout their vasculature greatly facilitate *in vivo* studies of vessel formation (Lawson and Weinstein 2002; Jin *et al.* 2005). In this study, the expression pattern and zebrafish embryonic development function of *TCONS_00028652* were explored.

Materal and Methods

Animal husbandry

Zebrafish (*Danio rerio*) were obtained from the Institute of Hydrobiology, Chinese Academy of Science (Wuhan, China). The transgenic *Tg* (*flil*:EGFP) (Friend leukemia virus integration 1) zebrafish line was from the Animal Center, Qixiu Campus of Nantong University, China. Embryos obtained from natural spawning of wild-type zebrafish were incubated and maintained at a light period of 14/10 h (light/dark) in accordance with described procedures (Westerfield 1995) and they were staged as previous report (Kimmel *et al.* 1995). All animal experiments were approved by the Institutional Animal Care and Use Committee at Henan Normal University. Embryos from wild-type AB strains were used for expression analysis, and transgenic strains [*Tg* (*fli1*: GEFP)] were used for functional analyses. Two male and one female fish were collected in mating cages the night before embryo collection. The embryos were staged according to hours post-fertilization (hpf). The developmental stages selected for this study were 0, 2, 6, 12, 24, 48, 60 and 72 hpf. For clearing whole embryos to observe gene expression, melanin pigment production was disrupted by raising embryos in 0.003% 1-phenl-2-thiourea (P3755; Sigma, St Louis, USA) before 24 hpf.

The identification and cloning of *TCONS_00028652*

We annotated *TCONS_00028652* using bioinformatics databases, such as Ensemble genome browser 98 (http://asia.ensembl.org/index.html), lncRNAdb (http://www.lncrnadb.rog/), and the UCSC genome browser (http://genome.ucsc.edu/). *TCONS_00028652* (ENSDART00000150571) located in chromosome 16 at position from15786804 to 15786237 and has only an exon (568 bp). *TCONS_00028652* (ENSDART00000150571) is a transcript of non-coding gene *si: dkey111b14.2* (ENSDARG00000096145). Cloned fragment was amplified, by designing primers against 5' and 3' exon-flanking sequences, and then sequenced by GENEWIZ biotechnology Co. Ltd. (Jiangsu, China). The cloned sequence was aligned with the *si: dkey111b14.2* sequence using NCBI blast in order to confirm the correct sequence. Then, we assessed its protein-coding potential using the online softwares: the Coding Potential Assessing Tool (CPAT) and the Coding Potential Calculator (CPC).

Quantitative real time PCR (qPCR)

Embryos gathered at various developmental stages were stored at –80°C until they were processed. Total RNA was extracted from 50 embryos at each stage using RNAiso Plus (TaKaRa Biotechnology Co., Ltd. China). With a Nanodrop-2000, total RNA content was calculated from absorbance at 260 nm and RNA purity was verified by the A_{260}/A_{280} ratio (> 1.8).

First strand cDNA was reversely transcribed from 1–5 μg total RNA with a HIFIScript 1st Strand cDNA Synthesis kit (Cwbiotich, China) used in accordance with manufacturer's instructions. Relative lncRNA expression levels were measured with qPCR and *β-actin* was used as the internal reference. The qPCR conditions were: polymerase activation for 10 min at 95°C and 40 cycles of 95°C for 10 s and 54–60°C for 30 s. The data were calculated using the $2^{-\Delta\Delta Ct}$ method. All primers were synthesized by GENEWIZ Biological Company (Suzhou, China) and are shown in Table 1.

Whole mount *in Situ* hybridization

In order to investigate the spatiotemporal expression pattern of *TCONS_00028652*, *in situ* hybridization with whole mount embryos was performed. Embryos were fixed in 4% paraformaldehyde. The *TCONS_00028652* PCR fragment was amplified from cDNA (Cwbiotich, China) and cloned into pGEM-T easy plasmid (Promega, USA). The construct was linearized with ApaI or NsiI. Digoxigenin-labeled antisense RNA probes were transcribed *in vitro* with Sp6 or T7 RNA polymerases using a DIG RNA labeling kit (Roche, Germany). *In situ* hybridization with zebrafish embryos at different stages was performed in accordance with the standard detection method (Thisse and Thisse 2007).

Morpholino injection

To investigate the function of *TCONS_00028652* in zebrafish embryonic vasculature development, we analyzed effects of *TCONS_00028652* loss of function on zebrafish embryonic development. Loss of function was created by

injecting antisense morpholino oligonucleotides (MO) into one-cell zygotes. One tactics would be to inject MOs targeted against lncRNA splice sites in an attempt to disrupt maturation. The other tactics would be to inject MOs designed against highly conserved regions supposed to be important to play its roles (Ulitsky *et al.* 2011). However, *TCONS_00028652* is a single exon lncRNA, thus targeting an lncRNA splice site was infeasible. Therefore, we designed MO with Gene Tools to target a highly conserved region of *TCONS_00028652*. The MO sequence was 5'-GCTTTTTTGATAACTCACCATGCCG-3', close to the 3' UTR. An equal volume of standard morpholino oligomers was used as a control. Morpholinos were dissolved in milliQ water as 1 m*M* stock solutions and diluted to 300 μM working solutions. For transgenic (*Tg*: *fli1*-GEFP) strains, 200 one-cell stage embryos were injected with about 5 nL of morpholino solution with a FemotoJet (Eppendorf). Zebrafish strain (*Tg*: *fli1*-EGFP) carries an endothelial-specific EGFP reporter that can visualize the details of developing blood vessels *in vivo*. Meanwhile, an equal number of one-cell-stage embryos were injected by standard morpholino oligomers, which do not specifically target genes in zebrafish embryos. Morphants were evaluated at 3 dpf with fluorescence microscopy.

Statistical analysis

The qPCR experiment was carried out in triplicate. Data are described as means ± SD. Comparisons between control and test were evaluated by one-way analysis of variance (ANOVA) using S.P.S.S. 17.0. Probability (P) < 0.05 was deemed as statistically significant.

Results

Identification of A novel lncRNA *TCONS_00028652*

A previous research identified *TCONS_00028652* as an embryo and adult heart enriched lncRNA (Wang *et al.* 2017), but its function is elucidate. In order to study its function, its real existence is firstly verified through proliferating a cloned fragment using cDNA of TCONS_00028652 as template by designing primers against 5' and 3' exon-flanking sequences (Fig. 1 c). The length of cloned fragment was 1092 bp (Fig. 1d). The results of sequencing and alignment confirmed its real existence. A lack of protein-coding potential was assessed using two online software: CPAT and CPC analysis (Fig. 1a, b). The same result was obtained using the NCBI ORF finder tool, which further confirmed that it does not encode protein.

Expression profile of LncRNA *TCONS_00028652* at different stages of zebrafish embryonic development

To clarify the expression pattern of *TCONS_00028652* during early zebrafish embryogenesis, we performed qPCR with embryos at eight developmental stages (2, 6, 12, 24, 36, 48, 60, and 72 hpf). The results showed that *TCONS_00028652* was expressed throughout embryonic development (Fig. 2i), with the highest expression at 6 and 12 hpf. The expression profile in zebrafish embryo indicates that *TCONS_00028652* may participate in zebrafish embryogenesis.

To some extent, gene function can be predicted by studying gene expression distribution. In order to confirm the spatiotemporal expression pattern of *TCONS_00028652* in embryos at different developmental stages, we executed whole mount *in situ* hybridization of *TCONS_00028652* using a digoxigenin-labeled antisense RNA. The *in situ* hybridization indicated that *TCONS_00028652* was highly expressed from fertilization through 72 hpf, highly consistent with the qPCR profile. This result suggests that *TCONS_00028652* may have a crucial role throughout embryonic development (Fig. 2). The early appearance of *TCONS_00028652* (0 hpf) raises the possibility of maternal origin or regulation.

In early stages (0, 2, and 10 hpf), *TCONS_00028652* expression was diffuse (Fig. 2a–c). However, in later stages (24, 36, 48, 60 and 72 hpf), it was expressed predominantly in brain and tail (Fig. 2d–h). Additionally, *TCONS_00028652* was expressed in other organs and tissues, such as muscle, heart, neural tissues, tail fin, and pectoral tissues, suggesting that *TCONS_00028652* might play multiple functions during zebrafish embryogenesis.

Morpholino knockdown of LncRNA *TCONS_00028652* and knockdown efficiency validation

In order to explore the potential role of *TCONS_00028652* in vasculature development, we created *TCONS_00028652* loss of function by injecting MO, an oligonucleotide targeting a conserved region of *TCONS_00028652* (Fig. 3e), into one- to two-cell stage *Tg (flil:EGFP)* transgenic zebrafish embryos. As a control, "standard morpholino" was injected, which does not target any zebrafish genes. The promoter for *Fli1* (Friend leukemia virus integration 1), is specifically expressed in hematopoietic and endothelial cells (Melet *et al.* 1996a), was applied to activate enhanced green fluorescent protein (EGFP) expression in all blood vessels throughout embryogenesis (Lawson and Weinstein 2002).

We tested the efficiency of synthesized *TCONS_00028652*-MO using qPCR and whole mount *in situ* hybridization. The results declared that *TCONS_00028652* expression was significantly reduced in *TCONS_00028652*-MO morphants compared with non-injected (NI) embryos and control-MO embryos at 48 hpf (Fig. 3).

Abnormal vascular and heart phenotypes in LncRNA *TCONS_00028652*-MO embryos

Table 1: Primers used for qPCR and *in situ* hybridization in this study

Primers	Sequences
TCONS_00028652-qPCR-F	GACACGGAAAGGATTGACAG
TCONS_00028652-qPCR-R	TTCGTTATCGGAATGAACCAG
TCONS_00028652-Probe-F	GAATACCGCAGCTAGGAA
TCONS_00028652-Probe-R	CGTTATCGGAATGAACCA
Fli1-Probe-F	TCGTCCCCGCAGACCC
Fli1-Probe-R	GACGCTGGGATTGGGGTAAA

F, forward, R, reverse

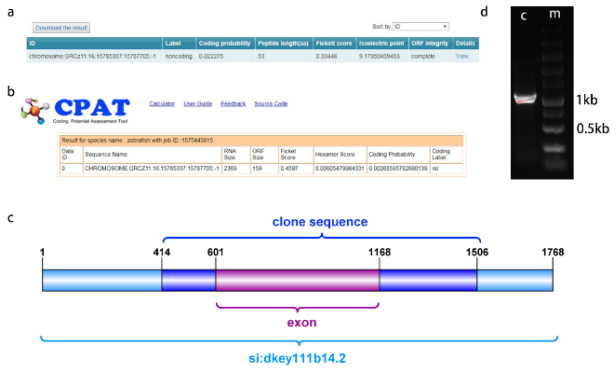

Fig. 1: Identification of *TCONS_00028652*

a: The protein-coding analysis using CPC; **b**: The protein-coding analysis using CPAT; **c**: The genetic structure diagram of *TCONS_00028652*; **d**: The agarose gel image of clone fragment; C: clone M: marker

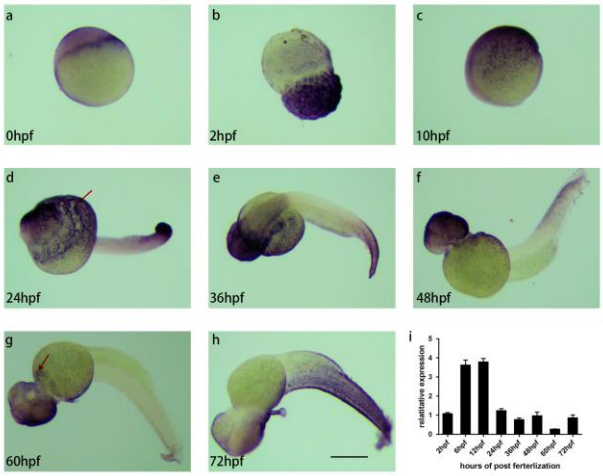

Fig. 2: Expression of *TCONS_00028652* during embryonic development in zebrafish

Scale bar=50 μm; **a-h**: *in situ* hybridiazation showing *TCONS_00028652* expression in zebrafish embryos at different stages of development (0, 2, 10, 24, 36, 48, 60, and 72 hpf); **i**: The relative expression of *TCONS_00028652* in zebrafish embryos at different stages of development; Red arrow points to the heart

Cardiac edema was observed in *TCONS_00028652*-MO morphants (Fig. 4f) compared with NI embryos and CON-MO embryos (Fig. 4e, g). Further, *TCONS_00028652*-MO embryos had significantly reduced body length (Fig. 4h). Previous reports have proposed a dynamic connection

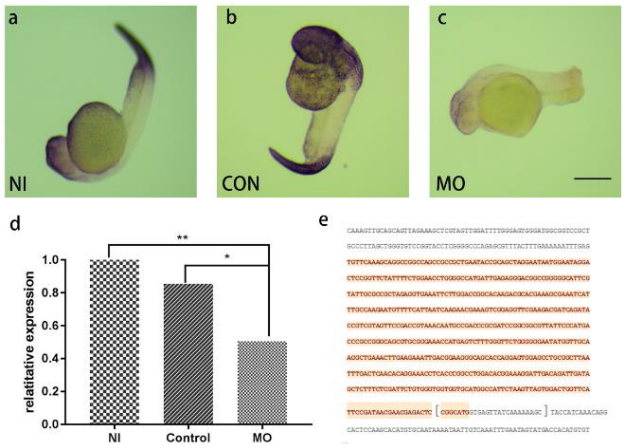

Fig. 3: Morpholino knockdown of *TCONS_00028652* in early zebrafish embryonic development

Scale bar = 50 μm; **a-c**: *In situ* hybridization showing the expression of *TCONS_00028652* from the *TCONS_00028652*-MO knockdown embryos or control-MO embryos (48 hpf); **d**: Relative expression level of *TCONS_00028652*; **e**: The target sequence showed by the bracket

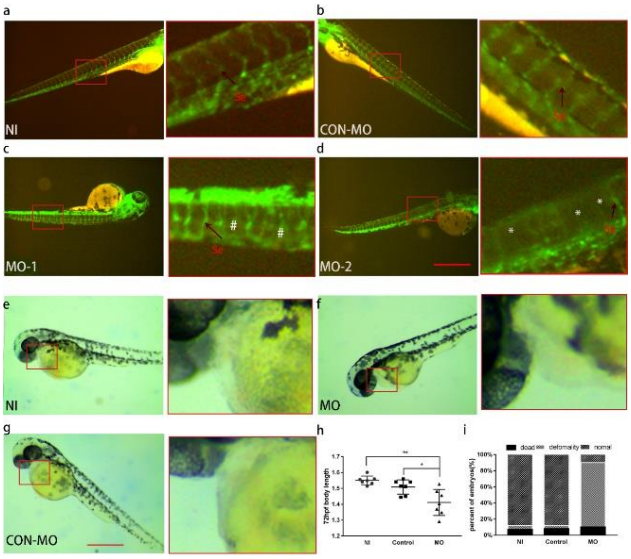

Fig. 4: Effect of *TCONS_00028652* knockdown on vasculature development and heart morphology in *Tg* (*fli1:* EGFP) transgenic zebrafish embryos

Scale bar = 50 μm; **a-d**: Fluorescent images of *Tg (fli1:* EGFP) at 3 dpf; #: points to the interruption of Se; *: points to the absent of vascular structures; The arrow points to the Se; **e-g**: The heart morphology in *TCONS_00028652*-MO embryos comparted compared to the NI and CON embryos; **i**: The quantity of the rate of deformities; NI: Non-injection; CON: Control-MO; Se: Intersegmental vessel

between somitogenesis and vasculature development (Torres-Vazquez *et al.* 2004; Mei *et al.* 2010). Thus, in order to determine if *TCONS_00028652* is essential for vasculature development, transgenic *Tg* (*fli1*:GEFP) embryos were injected with morpholino against *TCONS_00028652*, and vasculature development was evaluated. As shown in Fig. 4, compared with normal vasculature in NI embryos (Fig. 4a) and CON-MO embryos (Fig. 4b), the primary intersegmental vessel (Se) failed to

sprout and form in *TCONS_00028652*-MO embryos. Moreover, vascular structures were absent or abnormal (Fig. 4d) and extensive defects were observed in *TCONS_00028652*-MO embryos at 3 dpf (Fig. 4c). A higher rate of deformity was observed in *TCONS_00028652*-MO embryos (Fig. 4i). This result strongly indicates that *TCONS_00028652* knockdown can disturb intersegmental vessel development in zebrafish.

Fli1 expression in lncRNA *TCONS_00028652*-MO embryos with *in Situ* hybridization

To further verify the effect of *TCONS_00028652* knockdown on fish embryonic vascular development, whole-mount *in situ* hybridization was conducted in fish embryos, with endothelial cell marker *fli1* used as a monitor. The result showed that Se sprouts could be clearly observed in NI embryos (Fig. 5a) or CON-MO embryos (Fig. 5b); however, in *TCONS_00028652*-MO embryos, the corresponding vascular structure was absent or abnormal (Fig. 5c, d). Expression of *fil1* in lncRNA *TCONS_00028652*-MO embryos indicated missing endothelial cells (Fig. 5), which is consistent with the aberrant EGFP expression.

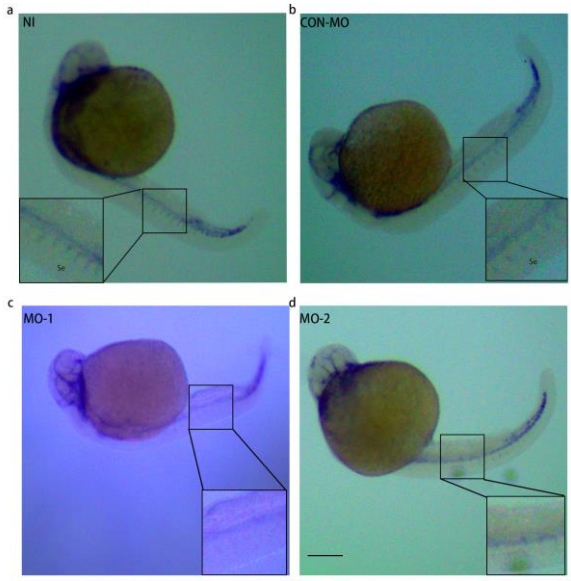

Fig. 5: *Fil1* expression in *TCNONS_00028652* -MO embryos as shown by *in situ* hybridization
Scale bar = 50 μm; **a**: NI embryos; **b**: CON embryos; **c** and **d**: *TCONS_00028652*-MO embryos; Black arrow points to the Se; NI: Non-injection; CON: Control-MO; Se: Intersegmental vessel

Discussion

In recent decades, with the employment of high-throughput deep sequencing approaches, the majority of long noncoding RNAs have been identified in many species, including zebrafish. Over 3000 lncRNAs have been identified in zebrafish embryos and adult tissues (Ulitsky *et al.* 2011; Pauli *et al.* 2012; Kaushik *et al.* 2013; Wang *et al.* 2017). However, the functions of most lncRNAs remain unclear.

Several reports now describe the function and molecular regulatory mechanism of lncRNAs in human diseases, especially cancer. However, evidence for lncRNAs function in animal development is still lacking. Deciphering the functional roles and regulatory mechanisms of development-related lncRNAs can reveal molecular mechanisms of diseases and also expand avenues for creating disease therapies. Recently, several development-related lncRNAs have been identified and characterized in zebrafish and mouse (Dinger *et al.* 2008; Pauli *et al.* 2015; Luo *et al.* 2016) and the results suggest that lncRNAs can substantially affect gene regulation during embryogenesis. For instance, lncRNA *tie-AS* was found to be involved in transcriptional regulation of vascular development (Li *et al.* 2009; Chowdhury *et al.* 2018). LncRNA *braveheart* was required for cardiovascular lineage commitment and activated the cardiac vascular gene network (Klattenhoff *et al.* 2013; Hou *et al.* 2017). LncRNA *fendrr* showed tissue-specific expression and was essential for proper heart and body wall development in mouse (Grote *et al.* 2013). Three other cardiovascular-related lncRNAs, *TERMINATOR*, *ALIEN* and *PUNISHER*, specifically expressed in undifferentiated pluripotent stem cells, cardiovascular progenitors, and differentiated endothelial cells, respectively, hence suggesting involvement in vertebrate cardiovascular development (Kurian *et al.* 2015). A novel lncRNA *durga* arising from the first exon of *Kalirin*, played a key role in axonal development, nerve growth and synaptic re-modeling, was reported to modulate dendrite density and *kalirin* expression in zebrafish (Sarangdhar *et al.* 2017). The sequence and central nervous system-restricted expression of lincRNA *TUNA* are strikingly conserved in vertebrates. Accordingly, *TUNA* knockdown in zebrafish impaired locomotor function, which suggests that lincRNA *TUNA* plays a vital role in pluripotency and neural differentiation in embryonic stem cells and is associated with adult vertebrate neurological function (Lin *et al.* 2014). In our study, lncRNA *TCONS_00028652* was generally expressed in early stages of embryonic development in zebrafish, predominantly in the embryonic brain, tail and heart. Knockdown of *TCONS_00028652* resulted in intersegmental vessel defects, suggesting that *TCONS_00028652* is indispensable for zebrafish embryonic vasculature development.

One effective strategy for pursuing gene function is to disrupt gene expression by gene knockdown or knockout. Gene knockout technology, such as CRISPR/Cas9, which is a widely used loss of gene function method, can alter the DNA gene locus. Alternatively, knockdown methods using morpholino oligonucleotides, a preferred zebrafish knockdown reagent made of 25 nucleotides substituting a morpholine ring and non-ionic phosphorodiamidate linkages for the ribose ring and phosphodiester backbone found in DNA and RNA (Mathew *et al.* 2019), can be used to

investigate lncRNAs (Li *et al.* 2009; Ulitsky *et al.* 2011; Goudarzi *et al.* 2019). MOs interfere with gene function by targeting either splice sites, in an attempt to disrupt lncRNA maturation, or highly conserved regions, presumed to be functional sites. MOs targeting splice sites or highly conserved regions in lncRNAs *Cyrano* and *Megamind* caused similar developmental defects (Ulitsky *et al.* 2011). In the present investigation of *TCONS_00028652*, only a highly conserved region could be targeted by MO because *TCONS_00028652* has only one exon and hence no splice sites. Our results showed that the knockdown by MO targeting the highly conserved region of *TCONS_00028652* caused developmental phenotype defects in embryonic blood vessels, as observed in Tg (*flil:EGFP*) transgenic fish expressing a cardiovascular marker gene.

Zebrafish are commonly considered a powerful model for studying genes and proteins regulating embryonic vascular development (Goishi and Klagsbrun 2004; McKinney and Weinstein 2008). A large amount of established techniques enable us to easily research zebrafish vessel development from early endothelial cell differentiation through adult vessel morphology (McKinney and Weinstein 2008). Transgenic zebrafish with GFP expression driven by the zebrafish *flil* promoter can be used to clearly visualize vasculature formation during zebrafish embryogenesis. *Fli1* is known as an endothelial cell marker in mouse (Melet *et al.* 1996b) and is also expressed during vascular development in zebrafish embryos (Thompson *et al.* 1998). In this study, transgenic zebrafish (*flil*:GEFP) facilitated the visualization of intersegment vessel defects induced by MO injection, proving their vascular-specific utility. Our results showed that embryonic heart and blood vessel defects could be observed clearly in *TCONS_00028652* morphants expressing the cardiovascular marker gene *fli1*.

Conclusion

The results of qPCR and whole mount *in situ* hybridization showed that lncRNA *TCONS_00028652* is generally expressed throughout early stages of embryonic development, predominantly in the embryonic brain, tail, and heart in zebrafish. Moreover, knockdown of *TCONS_00028652*, using morpholino oligonucleotides, resulted in intersegmental vessel defects, suggesting that *TCONS_00028652* is indispensable for zebrafish embryonic vascular development. However, the regulatory mechanism details of *TCONS_00028652* in embryonic intersegmental vessel development will further explore in future studies.

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Author Contributions

ZCE conceived the research and performed all experiments in this study. ZSQ provided transgenic zebrafish and valuable technical advice. MJG gave technical assistance. LXY provided research instruction throughout the study and reviewed the manuscript.

Conflict of Interest

There is no conflict of interest among the authors

Data Availability Declaration

All data, reported in this article are available with the corresponding authors and can be provided upon request

Ethics Approval

The guidelines for research on animals were duly observed

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