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Research article

Deficiency of *leap2* promotes somatic growth in zebrafish: Involvement of the growth hormone system

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ABSTRACT

Purpose: Liver-expressed antimicrobial peptide-2 (LEAP2) is identified as an endogenous antagonist and inverse agonist of the growth hormone secretagogue receptor type 1a (GHSR1a), its effect on the GHSR1a is contrary to the role of GHRELIN. Growth hormone (GH) is a crucial hormone for early development. Previous studies report that LEAP2 dose-dependently attenuates ghrelin-induced GH secretion, and Leap2-knockout mice exhibit increased plasma GH levels after GHRELIN administration. Clinical data revealed a possible correlation between LEAP2 and height development. However, the role of LEAP2 in early development remains unclear. This study aimed to investigate the role of LEAP2 in early development using *leap2* mutant zebrafish larvae as a model.

Method: We analyzed the conservation of LEAP2 peptide across multiple species and generated *leap2* mutants in zebrafish by CRISPR-Cas9, dynamically observed and measured the growth and development of zebrafish larvae from fertilization to 5 day post fertilization (dpf). In situ hybridization, transcriptome sequencing, quantitative real-time PCR and Western blot were used to detect the expression levels of GH and its signaling in early stage of embryonic development.

Result: Our data demonstrate that zebrafish with a knockout of the *leap2* gene display a significant increase in hatching rate, body length, and the distance between their eyes, all without visible developmental defects in the early stages of development. In addition, both RNA and protein analyses revealed a significant increase in GH expression in *leap2* mutant.

Conclusion: In general, this study demonstrates that LEAP2 regulates the expression of GH during early development, particularly influencing body length.

1. Introduction

Liver-expressed antimicrobial peptide 2 (LEAP2), a 40-amino acid secretory peptide with two disulfide bonds, is primarily expressed in the liver and jejunum in both rodents and humans [1–3]. LEAP2 orthologs are highly conserved across mammals,

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including rhesus monkeys, bovines, and mice [1], and originally identified as an antimicrobial peptide [1]. However, subsequent investigations revealed that the effective concentration of LEAP2 is approximately 3000-fold higher than its physiological concentration in human plasma [1,2,4]. In 2018, Xuecai Ge and colleagues advanced our understanding of LEAP2 by discovering a significant increase in *Leap2* mRNA expression in the stomach and a simultaneous decrease in the duodenum after vertical sleeve gastrectomy in high-fat diet-induced obese mice [2]. This finding was pivotal in unraveling the biological role of LEAP2. Subsequently, LEAP2 has been identified as the main endogenous antagonist/inverse agonist for GHSR1a, a G protein-coupled receptor whose endogenous agonist is GHRELIN [2,5].

Recent studies have highlighted the effects of both normal and disrupted LEAP2/GHRELIN balance on energy homeostasis. Plasma LEAP2 levels fluctuate inversely with GHRELIN based on energy status: LEAP2 levels increase in individuals with energy surplus (such as obesity or food intake) and elevated blood glucose, while they decrease during energy deficit (such as fasting or weight loss) [6]. LEAP2 binds to GHSR1a, effectively blocking the major effects of GHRELIN, including GHRELIN-induced hyperphagia, improved cognitive, blood glucose elevation, activating arcuate NPY neurons and body temperature in rodents [2,6]. Conversely, antibody neutralization of LEAP2 and *Leap2* knockout (KO) enhanced GHRELIN action in mice. Leap2-KO mice fed with a high-fat feeding (HFD) exhibited lower energy expenditure, increased food intake, greater body weight, lean mass, and hepatic fat, compared to their littermates on the same diet [7]. Disrupted LEAP2/GHRELIN balance is also associated with various diseases, including gambling disorder [8], anorexia nervosa [9], amyotrophic lateral sclerosis [10], nonalcoholic fatty liver disease [11], and age-associated cognitive decline [12].

GHRELIN is a brain-gut peptide primarily synthesized and secreted by gastric X/A-like cells. By binding to and activating its receptor GHSR1a, GHRELIN stimulates the secretion of growth hormone (GH), establishing it as one of the most potent GH secretagogues identified [13]. GH plays a key physiological role in longitudinal growth and metabolism [14]. Its importance becomes apparent in infancy and continues throughout childhood and adolescence, with GH deficiency in children resulting in dwarfism [15]. Our previous studies have shown that *ghrelin* primarily regulates the initiation of *gh* expression during zebrafish adenohypophysis development. Depletion of *ghrelin* function dysregulated *gh* and related-signaling and causes defects in zebrafish embryonic development. Notably, GH can partially rescue phenotypes [16,17]. Recent studies indicate that LEAP2 blocks GHRELIN-induced GH secretion, and blocking endogenous LEAP2 enhances fasting-induced GH release. Leap2 KO mice exhibit enhanced GHRELIN-induced GH secretion, and female Leap2 KO mice on a high-fat diet have greater body length than their wild-type littermates [7]. This suggests that LEAP2 may regulate GH secreting. However, the role of LEAP2 in early embryonic development and GH expression remains largely unknown. Therefore, this study aimed to explore the function of LEAP2 in early development and its regulation during this critical period.

2. Method

2.1. Animals

Adult zebrafish, aged 6–8 months, were raised at 28 ± 1 °C with a 14-h light cycle and a 10-h dark cycle and were fed saltwater shrimp. Zebrafish larvae were cultured using E3 solution at a temperature of 28 ± 1 °C. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Wenzhou Medical University.

Images of zebrafish larvae were taken using a stereomicroscope (Olympus Corporation, Japan). These images were used to observe

Table 1
Primer list.

Gene name	Sequence(5′-3′)
leap2 genotype F	TGCTGACTGGAGGGTATATAAGA
leap2 genotype R	ATGGACGCAGATGCTGATAAAG
***leap2* F**	ATCCAAGGGCTCGGCTCCATC
***leap2* R**	GATCTCGGCGTATCTGGTCAACATG
plcb3 F	TCAAACGGGGGAGCAAGTTT
plcb3 R	AGGATTTCCGCTTCCATGCT
itpr1b F	ACTAGACGCCGCGATTTTCA
itpr1b R	CCACTTTGTGTCGTGCCTTC
fosb F	AAGGGATGATGCAGGAGAGGGA
fosb R	ATAGGCAAGCAAAAGGCCGAG
raf1b F	TCCATTCTGTGGATGGCTCC
raf1b R	TTCTGGATGCAGTCGGACAC
adcy1a F	GTGGAGCCAGGATTTGGTCA
adcy1a R	AGCCCAGGAAAAATCTTGCG
atf2 F	ACTACTCACTGATGACAAGGAGG
atf2 R	AGTTGGCCAGAAGCACATTG
pou1f1 F	AACTCACTGCAAGGAGCTCTG
pou1f1 R	GGGCTCATCTGTTAGCAGGG
itpr2 F	TAACCTGGTGTGTGAGACGC
itpr2 R	GCCTGGCTATGCATGACTGA
ef1-α F	CTGGAGGCCAGCTCAAACAT
ef1-α R	ATCAAGAAGAGTAGTACCGCTAGCATTAC

and calculate various aspects of zebrafish larvae growth and development, such as survival, hatching, and deformities. MeizsMcs image analysis software was used to measure body length and the distance of eyes.

2.2. Leap2 knockout

CRISPR-Cas9 technology was employed to generate knockout of the leap2 gene in zebrafish. Genotype-specific guide RNA (sgRNA) was designed to target the optimal CRISPR site of the leap2 gene [18]. The sgRNA targeting site is located on the first exon of leap2. The protospacer adjacent motif (PAM) is 5′ GTTTCTGCTGATTGTCCAACAGG 3'. After transcribing the sgRNA at the target site, Cas9 mRNA were mixed and co-injected into zebrafish embryos. The final concentrations of Cas9 mRNA and gRNA were 100 ng/μL and 150 ng/μL, respectively.

2.3. Genotype examination

Genotype detection was performed using genomic DNA as a template for PCR, followed by agarose gel electrophoresis and GoldView staining for imaging [19]. The PCR products of *leap2*$^{+/+}$ and *leap2*$^{-/-}$ zebrafish are single band with sizes of 371 bp and 367 bp, respectively, while *leap2*$^{+/-}$ zebrafish showed a double band. Primers used for genotype detection of leap2 are listed in Table 1. The amplified product of *leap2*$^{+/+}$ zebrafish was cut into two fragments of 115 bp and 257 bp restriction endonuclease *Mme*I, while the product of *leap2*$^{-/-}$ zebrafish remained uncut.

2.4. Behavioral experiments

The locomotor activity analysis of zebrafish larvae was conducted as described previously [20]. Briefly, 5 dpf zebrafish larvae were transferred individually to each well of a 96-well plate, and then placed in the behavioral test chamber for 30-min acclimation period. An automated video tracking system (Videotrack; Viewpoint Life Sciences, France) was used to monitor behavioral activity for 2 h and 20 min, recording the movement of each larvae every 30 s. The locomotors activity analysis protocol was as follows: 1 h of light phase (10 % illumination, 800 lux), 1 h of dark phase (0 illumination, 0 lux), and light stimulation (100 % illumination, 8000 lux) in 10 cycles of 1 min on and 1 min off, lasting a total of 20 min.

2.5. Whole mount in situ hybridization

The whole-mount *in situ* hybridization (WISH) protocol was based on our previous studies [21]. First, zebrafish larvae were fixed using 4 % (wt/vol) paraformaldehyde at 4 °C overnight. Next, the fixed larvae were placed at −20 °C in 100 % methanol for at least 2 h before use. The larvae were then rehydrated in a methanol gradient, washed with phosphate-buffered saline containing 0.1 % Tween 20 (PBST), permeabilized with 10 μg/mL proteinase K (Roche, #03115879001), and incubated with digoxigenin-labelled RNA probes overnight at 65 °C. The next day, after removing the probe, the larvae were incubated at 65 °C in a gradient concentration sodium citrate (SSC) hybridization buffer and in a 1:5000 dilution of anti-digoxigenin-alkaline phosphatase (Roche, #11093274910) overnight at 4 °C. Finally, after washing 6 times with PBST (15 min each time), NBT/BCIP (Roche, 11681451001) was diluted 1:50 in alkaline tris buffer (100 mM Tris HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1 % Tween 20) and incubated at room temperature in the dark for color development. When an appropriate signal appeared, the reaction was terminated, and the larvae were imaged using an Olympus microscope (Olympus, Tokyo, Japan). The pixel values of WISH signals were measured and quantified using National Institute of Health (NIH) ImageJ analysis software.

2.6. RNA extraction and cDNA synthesis

Total RNAs were isolated from 25 embryos of each group using RNAiso Plus, following the animal tissue procedure (TaKaRa, catalog #9108). The RNA concentration was determined using a Nanodrop (Thermo scientific) and then reverse-transcribed into cDNA using PrimeScript™ RT Master Mix (TakaRa, No. RR036Q).

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was performed using QuantStudio™ Dx Real-Time PCR Instrument and ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711-03). Reaction mixtures were prepared in triplicate according to the manufacturer's protocol. Expression levels of *leap2*, *gh* and GH signaling pathway-related genes, including *plcb3*, *itpr1b*, *fosb*, *raf1b*, *adcy1a*, *atf2*, *pou1f1* and *itpr2* were analyzed. The housekeeping gene *ef1α* was used as an internal control. Primer sequences are listed in Table 1. Quantification was conducted using the $2^{-\Delta\Delta Ct}$ method [22].

2.8. Transcriptome sequencing

The *leap2*$^{+/+}$ and *leap2*$^{-/-}$ zebrafish larvae at 2 dpf and 4 dpf were collected, and total RNA was isolated using RNAiso Plus (TaKaRa, catalog #9108). The procedures for sample quality assessment, RNA library preparation, and RNA sequencing (RNA-seq) were conducted as previously described [17]. Library construction and high-throughput RNA-sequencing were performed by

GENEWIZ (Suzhou, China)

2.9. Western blot

After removing the yolk from 25 larvae, the samples were ground using RIPA lysis solution to extract total proteins. The protein samples were then separated by SDS-PAGE, transferred to a PVDF membrane, and blocked in 5 % skim milk for 1 h at room temperature. The membrane was incubated with zebrafish gh antibody (Wuhan DIA AN Biotechnology Co., LTD) overnight at 4 °C. After washing the next day, the membrane was incubated with a peroxidase-conjugated secondary antibody at room temperature for 1 h. ECL chromogenic solution was used to develop signals, and ImageJ was used to quantify the grayscale values of the bands.

2.10. Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.5. The data were analyzed using Student's *t*-test. Data are expressed as mean ± SEM. The probability of $p < 0.05$ is statistically significant.

3. Result

3.1. Conservation of LEAP2 genes and generation of *leap2*$^{-/-}$ zebrafish

To determine whether the loss of protein function in zebrafish could mimic the human phenotype, we first analyzed the conservation of LEAP2 peptide across multiple species. MEAG 7 software was used to construct an evolutionary tree for the mature LEAP2 peptide sequences of the 20 species (Fig. 1A). The results showed that zebrafish and mice are closely related. As shown in Fig. 1B, the leap2 gene encoding in zebrafish, mouse, rat and humans contains a 63-aa conserved amino acid conserved region (data from other more species is not shown). Protein sequence alignment of mature LEAP2 from *H. sapiens*and *D. rerio* shows that the mature peptide of LEAP2 is highly conserved between humans and zebrafish, especially in the N-terminal region, which is essential for binding of agonist and the antagonist of GHSR1a [5], suggesting mature peptide of LEAP2 is highly conserved in function (Fig. 1C).

We then investigated the expression level of *leap2* mRNA at various early stages of zebrafish embryo development, qRT-PCR results indicated the presence of *leap2* mRNA in maternal RNA at 0 hour post-fertilization (hpf), with expression peaks at both 24 hpf and 96 hpf (Fig. 1D).

To explore the physiological role of leap2, we used CRISPR/Cas9 technology to generate *leap2* mutants in zebrafish (Fig. 1E). After screening, we obtained zebrafish lines with nucleotide changes. Sanger sequencing and PCR confirmed that 4 base pairs were deleted from the mutant zebrafish leap2 nucleotide sequence (Fig. 1F–H). Bioinformatic predictions suggested that these genomic alterations lead to frameshifting and premature termination of protein translation, resulting in a deficiency of LEAP2 (Fig. 1I and J).

3.2. *leap2*$^{-/-}$ larvae exhibited accelerated development

To study the impact of *leap2* deletion on zebrafish, the growth, development, survival, and reproduction of zebrafish were monitored. The survival rate of *leap2*$^{-/-}$ zebrafish larvae was not significantly different from that of the *leap2*$^{+/+}$ zebrafish, and adult zebrafish can reproduce normally. Interestingly, *leap2*$^{-/-}$ zebrafish larvae exhibited increased hatchability at 2 dpf (Fig. 1A and B) and increased body length, distance between the eyes (reflecting head size) and eye size at 3–5 dpf (Fig. 2C–F). However, no other obvious morphological abnormalities, such as pericardium edema, smaller eyes, body color change and tail bending were observed (data not shown), suggesting that somatic growth and development of *leap2*$^{-/-}$ zebrafish larvae are accelerated.

Although *leap2*$^{-/-}$ zebrafish larvae grew and developed faster than *leap2*$^{+/+}$ zebrafish, the locomotor behavior analysis showed that their movement ability and visual sensitivity did not change (Fig. 3), both in the regular phase (light phase and dark phase) and under light/dark cycle stimulation phase.

3.3. *leap2*$^{-/-}$ larvae exhibited increased gh expression

Since *leap2*$^{-/-}$ zebrafish larvae had faster somatic growth than *leap2*$^{+/+}$ zebrafish larvae, the expression level of gh, a key gene in embryonic growth, was evaluated. The qRT-PCR and WB results showed that the expression of gh in *leap2*$^{-/-}$ zebrafish larvae during 3-5dpf was significantly up-regulated (Fig. 4B and C). Similarly, WISH found that *gh* expression increased in the pituitary region. (Fig. 4A). The increase in gh expression may contribute to the increased body length of *leap2*-deficient zebrafish larvae.

3.4. The mRNA levels of growth hormone-related genes increased in leap2 mutant larvae

Given the significant increase in incubation rate observed for *leap2*$^{-/-}$ embryos at 2 dpf and the expression peak of *leap* mRNA at 4 dpf in zebrafish embryos, we selected zebrafish larvae at 2 dpf and 4dpf for transcriptome analysis to further elucidate the molecular mechanisms involved in *leap2* depletion during zebrafish embryonic development. Results showed changes in genes related to the *gh* signaling pathway at both 2dpf and 4dpf. (Fig. 5A). qRT-PCR verification found that the expression of *plcb3* and *itpr1b* showed an upward trend (Fig. 5B and C), while the expression of *raf1b*, *adcy1a*, *atf2*, *pou1f1* and *itpr2* increased significantly (Fig. 5E–I). Conversely, *fosb* was significantly down-regulated (Fig. 5D). These results indicate the activation of the *gh* signaling pathway in *leap2*$^{-/}$

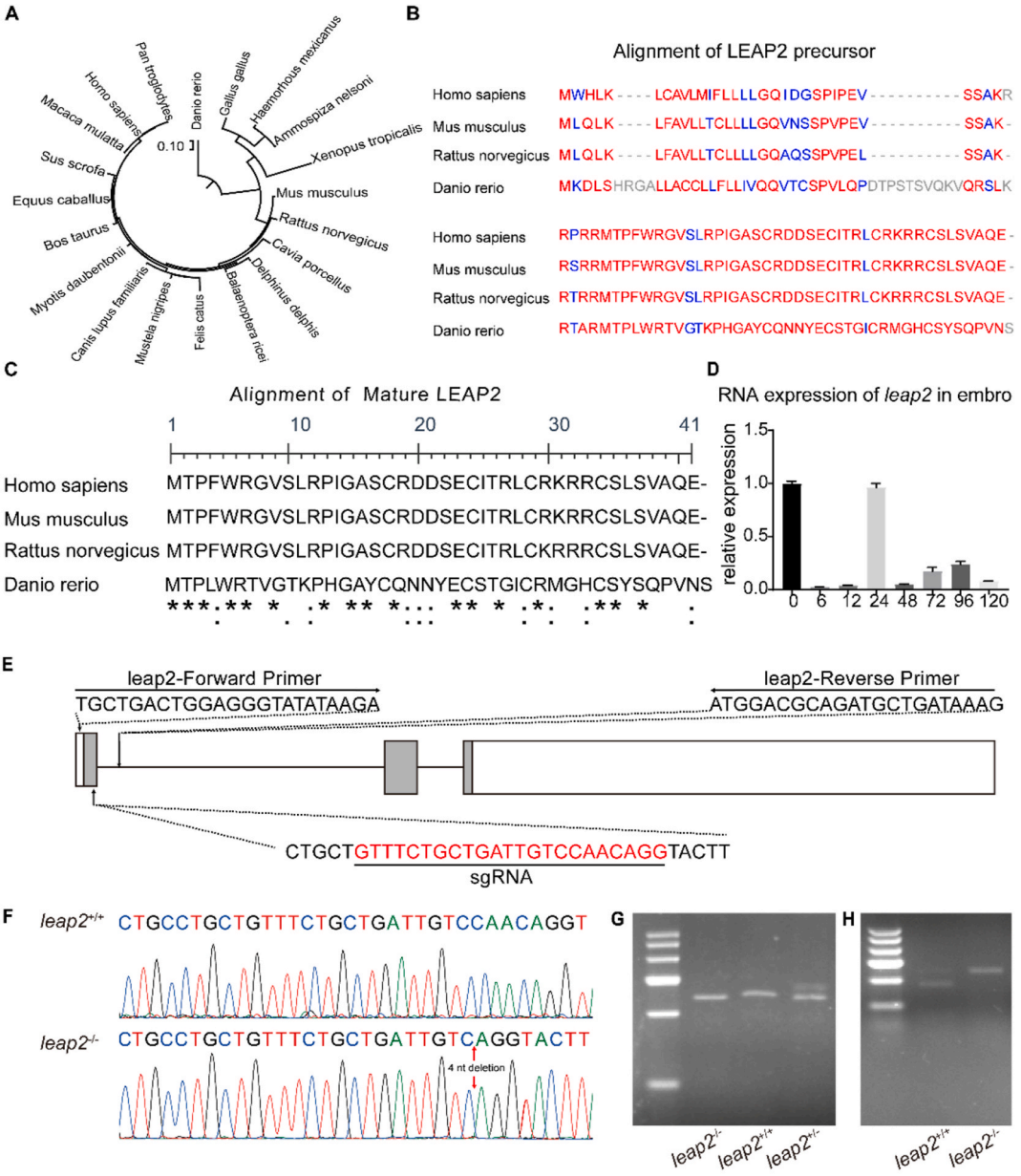

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Fig. 1. Generation of *leap2*-deficient zebrafish.
(A) Phylogenetic tree of LEAP2. (B) Multiple sequence alignment columns with no gaps are colored in blue or red of LEAP2 precursor. The red color indicates highly conserved columns and blue indicates less conserved ones. (C) Alignment of mature LEAP2, complete conservation is marked with "*", molecules with roughly similar sizes and the same hydrophilicity and hydrophobicity are marked with ":", and residues whose molecular size and hydrophobicity are preserved to a certain extent are marked with "." (D) Relative expression levels of *leap2* in various stages of zebrafish embryos. (E) Specific guide RNA (sgRNA) site is targeted in the first exon of leap2. (F) Electropherogram of Sanger DNA sequencing for *leap2*$^{+/+}$ and *leap2*$^{-/-}$ zebrafish. (G) The gel electrophoresis of PCR products spanning mutated sites from mutant (−/−), wild type (+/+) and heterozygous (±). (H) The PCR products of *leap2*$^{+/+}$ and *leap2*$^{-/-}$ zebrafish were digested by *Mme*I. (I) Comparison of DNA sequences identified in *leap2*$^{+/+}$ and *leap2*$^{-/-}$ zebrafish lines. (J) The alignment of predicted protein sequences in *leap2*$^{+/+}$ and l *leap2*$^{-/-}$ zebrafish lines. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

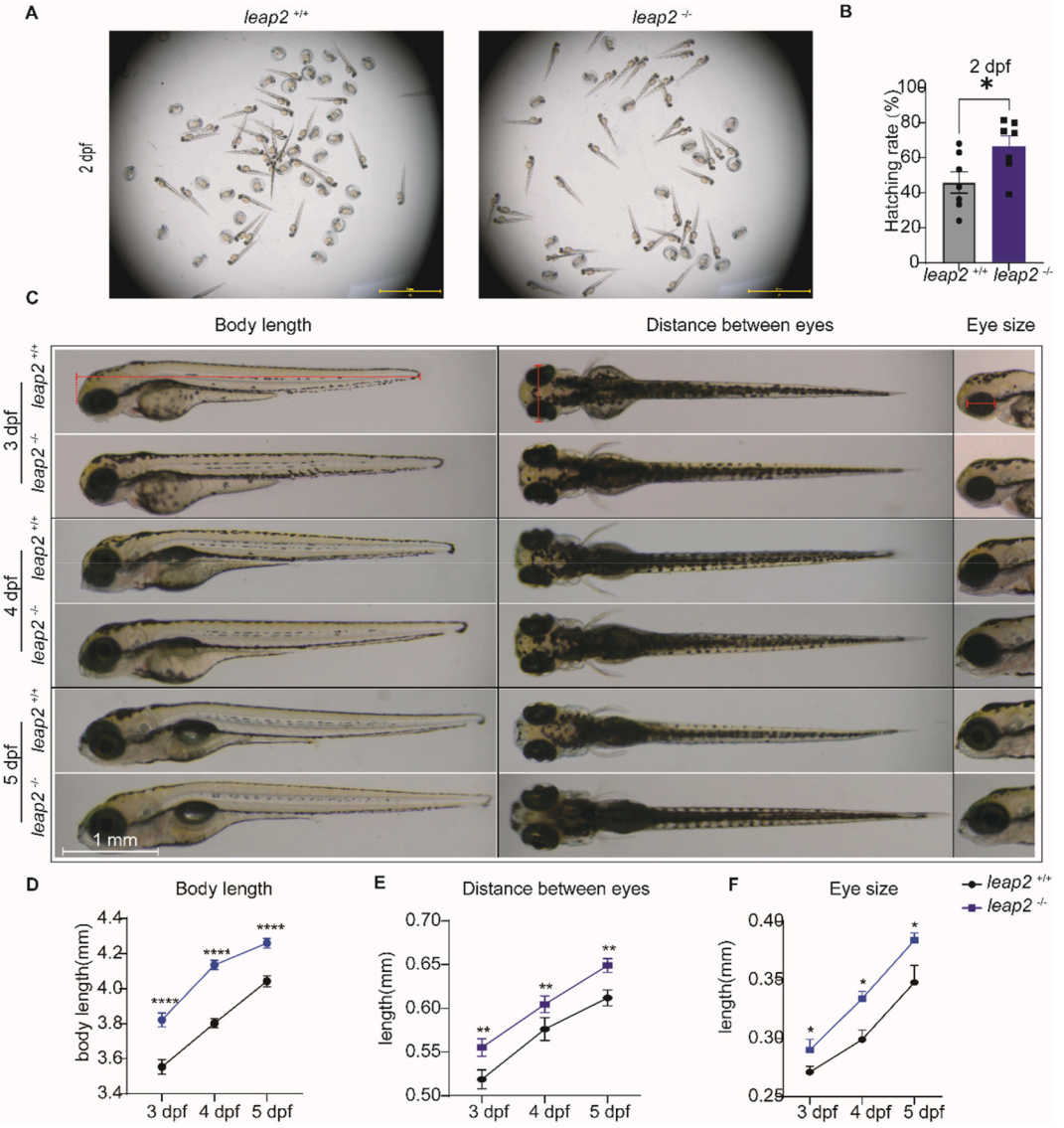

Fig. 2. The growth and developmental process of *leap2*$^{-/-}$ mutant zebrafish is accelerated.
(A) Representative image of embryo hatching and hatchability at 2 dpf, scale bar = 5 mm. (B) Hatching rate of 2 dpf *leap2*$^{+/+}$ and *leap2*$^{-/-}$ zebrafish larvae. (C) Representative picture of whole body of zebrafish larvae (side view), zebrafish larvae (top view) and eyes. (D) Body length of 3 dpf, 4 dpf, 5 dpf zebrafish larvae, (E) the distance between eyes and (F) Eye size of zebrafish larvae was measured, scale bar = 1 mm, Data analysis was performed using Student t-test, *p < 0.05 **$p < 0.01$, ****$p < 0.0001$.

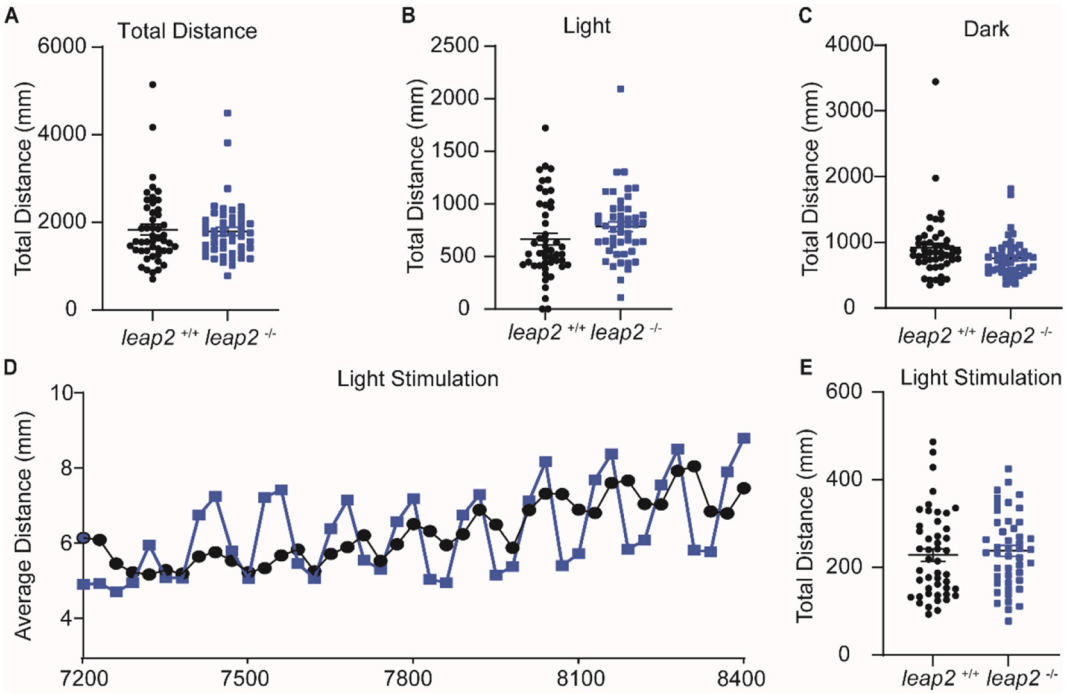

Fig. 3. Locomotor behavior of 5 dpf zebrafish larvae.
Total locomotor activity distance in 2 h and 20 min(A), 1-h light phase (B), 1-h dark phase (C). (D) Behavioral patterns under 20 min of light/dark cycle stimulation. (E) Swimming distance under light/dark cycle stimulation. N = 48. Data analysis was performed using Student t-test.

$^{-}$ embryos.

4. Discussion

The advantages of in vitro fertilization and embryonic transparency make zebrafish an excellent model for vertebrate developmental biology and neurology in the early life stage [23,24]. This study utilized *leap2*$^{-/-}$ larvae to evaluate the endogenous function of LEAP2 during early embryonic development. The novelty of the present study lies in the observation that loss of *leap2* in larval zebrafish resulted in accelerated somatic growth, including hatching, increased body length, eye size, and distance between eyes at 3–5 dpf. Through WISH, WB, RNA-seq analysis, and qRT-PCR verification, we found that *leap2* deficiency in zebrafish promoted the expression of *gh* and downstream genes.

LEAP2 is relatively conserved during evolution, particularly in fish where it is a more conserved GHSR1a ligand than GHRELIN [25]. The *leap2* gene encodes precursors of LEAP2, which include an N-terminal signal peptide, a brief propeptide, and a mature peptide. Protein sequences alignment revealed that the zebrafish leap2 precursor contains a much longer propeptide and the similarity between the zebrafish leap2 precursor and the human LEAP2 precursor is about 43 %. This variation is mainly due to differences in the signal peptide and brief propeptide regions. Compared to signal peptides and propeptides, the mature peptide shares high sequence homology between zebrafish and human. The human LEAP2 mature peptide comprises 40 amino acid residues, whereas the zebrafish counterpart contains 41. Notably, the N-terminal residues Trp5 and Arg6 play a key role in the binding of human LEAP2 to its receptor [26], and these residues are also present in the zebrafish leap2 mature peptide. Both the C-termini of the mature LEAP2 peptide proteins in zebrafish and human contain two characteristic cysteine residues that participate in the formation of disulfide bonds [27]. Additionally, the C-terminus of zebrafish leap2 boasts an extra amino acid, Selenocysteine. This unique residue, known for its antioxidant properties, suggests a potential enhancement in the zebrafish's innate immune arsenal, which may be species-specific to zebrafish. Overall, these findings suggest the high conservation of LEAP2 mature peptide between humans and zebrafish. This animal model in our study would be invaluable for delving deeper into the mechanisms of endogenous LEAP2 in human.

In a treatment study involving boys with constitutional delay of growth and puberty (CDGP), a negative correlation between growth velocity and circulating LEAP2 level was revealed [28]. A cross-sectional pilot study showed a trend toward higher LEAP2 levels in adult GH deficiency patients, though the difference was not significant [29], implying an associated LEAP2 and height. Our study is the first to report that *leap2*$^{-/-}$ larvae displayed greater body length than *leap2*$^{+/+}$ larvae, elucidating the regulatory effect of LEAP2 on height in the early stage. This is a surprising phenotype, as GHRELIN's promotion of GH secretion has been widely reported [30], However, at the endogenous level, transgenic mice overexpressing GHRELIN or its analog did not exhibit observed increases in GH and body length compared to controls [31–33]. This discrepancy puzzled scientists until the discovery of the counter-regulatory effect between LEAP2 and GHRELIN, which provided further clues [34]. LEAP2 functions as a robust antagonist and an inverse agonist

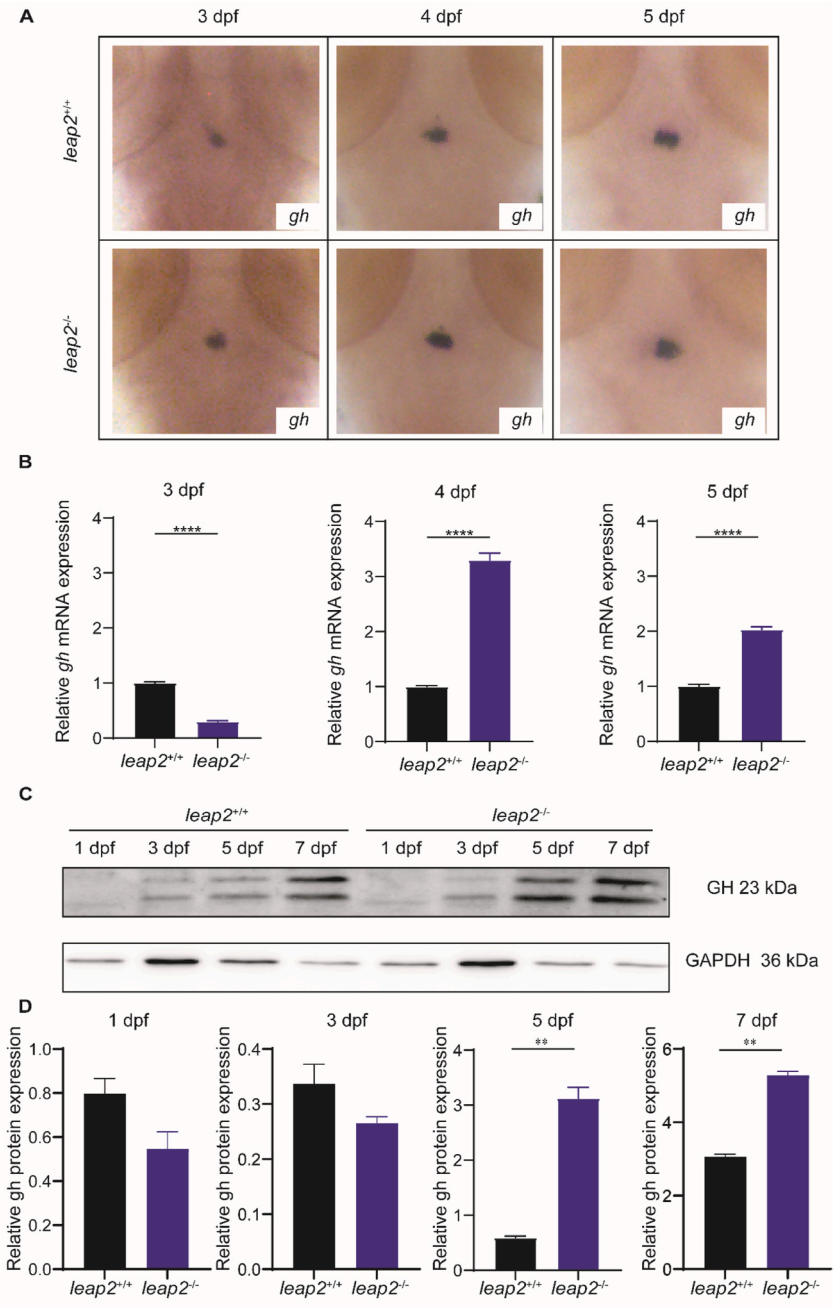

Fig. 4. Deletion of *leap2* results in increased *gh* expression in zebrafish larvae.
(A) Representative images whole-mount zebrafish embryo *in situ* hybridization. (B) Relative expression of *gh* in 3 dpf, 4 dpf and 5 dpf of zebrafish larvae. (C) Western blot detection GH protein expression. (D) Relative gh protein expression of zebrafish larvae. Data analysis was performed using Student t-test. ****$p < 0.0001$.

of GHSR1a, neutralizing its constitutive activity [35]. LEAP2 can inhibit the release of GH, and in Leap2 KO mice, the actions of ghrelin as a GH secretagogue are enhanced [2,7]. Pre-treatment with LEAP2 rendered brain slices unresponsive to the addition of GHRELIN, preventing any changes in the membrane potential of NPY neurons [6]. GH is secreted by the anterior pituitary, *in situ* hybridization highlights the *gh* expression in the pituitary region was elevated in *leap2*$^{-/-}$ larvae. Western blot and RNA-seq revealed an increase in both protein and mRNA expression levels, triggering a series of downstream factors of GH signaling pathways in including *plcb3*, *itpr1b*, *raf1b*, *adcy1a*, *atf2*, *pou1f1* and *itpr2* in *leap2*$^{-/-}$ larvae. The increase in GH and activation of downstream pathways, combined with the increase in body length in *leap2*$^{-/-}$ larvae, provide encouraging evidence supporting the hypothesis that LEAP2 is the key factor in GHRELIN resistance [7]. This discovery offers a potential target for intervention of early height development.

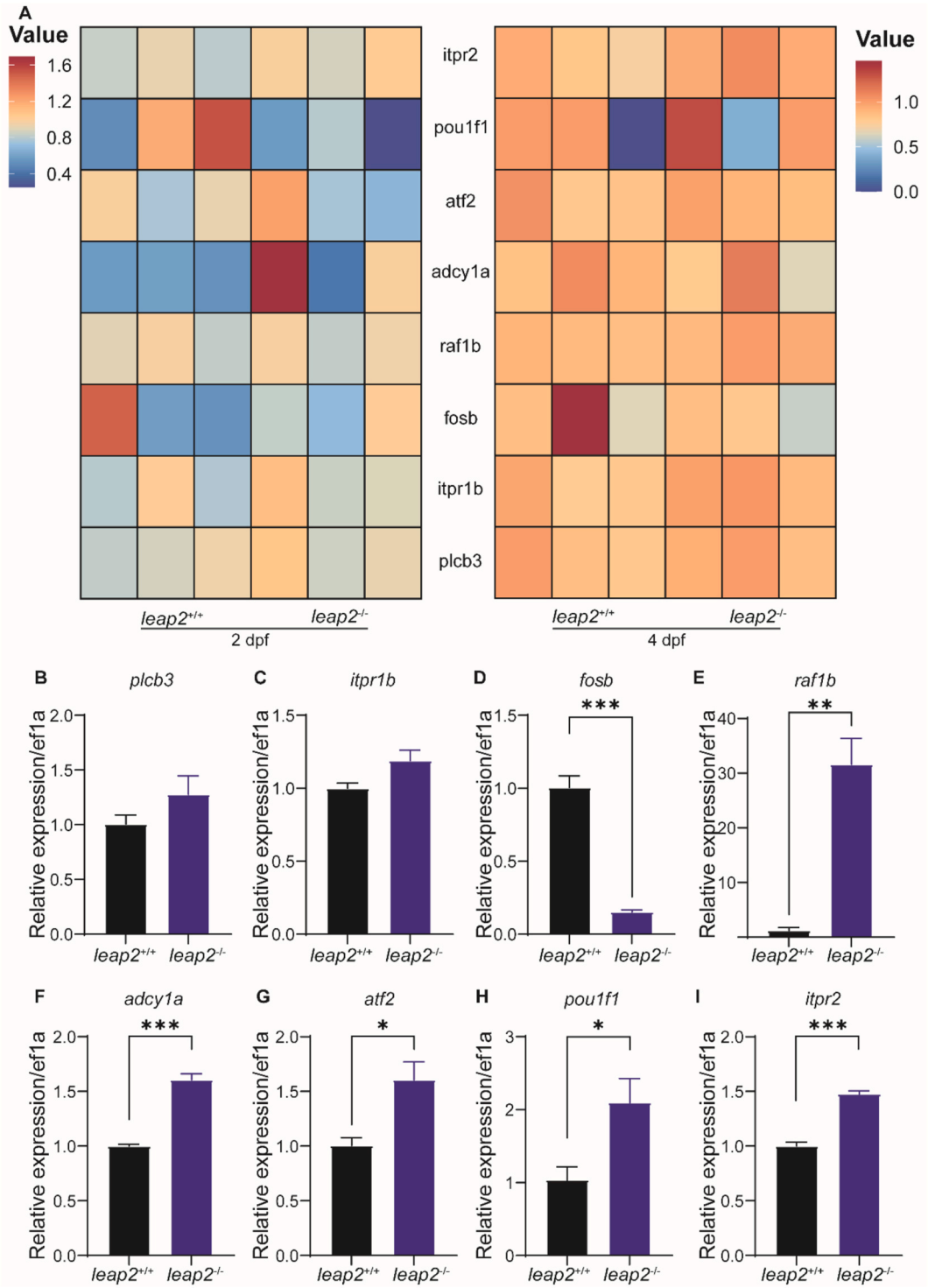

Fig. 5. Changes in GH signaling pathway in *leap2*-deficient zebrafish larvae.
(A) Heat map of gene expression related to *gh* signaling pathway by transcriptome sequencing. (B–I) Relative expression levels of genes related to *gh* signaling pathway in 5 dpf zebrafish larvae. Data analysis was performed using Student t-test. *$P < 0.05$, **$P < 0.01$, ****p < 0.0001.

Our previous study showed knockdown of endogenous *ghrelin* in zebrafish larvae by microinjection of morpholinos resulted in smaller eyes, shorter body length, air sacs deficiency, and pericardial edema compared to control [16]. Conversely, *leap2*$^{-/-}$ larvae displayed accelerated hatching, increased body length, eye size and distance between eyes without visible developmental defects.

Although possibly due to compensation response, *ghrelin* knockout did not exhibit these morphological defects phenotype in *ghrelin* knockdown did not exhibit these morphological defects but showed a neurodevelopmental disorder, specifically an Attention Deficit/Hyperactivity Disorder (ADHD)-like phenotype and impaired dopaminergic system development [19]. Emilio R. Mustafá and colleagues reported LEAP2 impairs GHSR1a regulation of dopamine 2 receptors signaling on voltage-gated calcium channels type 2.2 currents [36]. An increase in LEAP2 has also been found to contribute to age-associated cognitive decline [12], implying its potential role in neurological function. In terms of morphology, we observe that *leap2*$^{-/-}$ displayed a significantly increased head size (the distance between eyes of larvae) and eyes. Both smaller and larger head/eyes may have problems with the development of the nervous system. Zebrafish larvae typically exhibit a preference for dark environments and become hyperactive (visuomotor responses) when transitioning from black to light conditions [37]. We assessed the swimming behavior of *leap2*$^{-/-}$ larvae under different illumination condition and cycles to evaluate visual sensitivity and response to external stimuli. *Leap2*$^{-/-}$ larvae did not exhibit any abnormalities in avoidance reflex, visual sensitivity or locomotor behavior. Further neurobiological research is needed to explore the role of LEAP2 in the nervous system.

qRT-PCR revealed that *leap2* mRNA was present in maternal RNA (0 hpf) and showed expression peaks at 24 hpf and 96 hpf, then gradually decreased. Our previous research found that *ghrelin* mRNA can be detected from 12 hpf and gradually increased thereafter [16]. We predict that this difference indicates a reduction in LEAP2's resistance to GHRELIN and enhances the effect of ghrelin on *gh* expression during very early development. The phenotype of accelerated hatching after knockout of *leap2* seems to be consistent with this prediction, but further research is needed.

Sex-dependent dimorphic effect of the GHRELIN/GHSR1a system on pulsatile GH secretion has been reported [38,39]. A human study reports that girls exhibit higher concentrations of plasma LEAP2 than boys [40]. Additionally, boys with CDGP undergoing treatment with aromatizable testosterone exhibited a negative correlation between changes in LEAP2 levels and changes in estradiol levels [28]. Leap2$^{-/-}$ mice on a HFD exhibited longer body length than their wild-type littermates, but this was not observed in male Leap2$^{-/-}$ mice [7]. These findings indicate sexual dimorphism in the function of LEAP2. The gonads of zebrafish larvae remain undifferentiated and are bi-potent until approximately 12 dpf [41]. In *leap2*$^{-/-}$larvae during 3-5dpf, we can reasonably exclude the interference of gender and sexual hormones, providing a more accurate and specific understanding of the function of leap2 in growth.

In conclusion, the mature peptide of LEAP2 in zebrafish is highly conserved to humans and is expressed during early development, suggesting that leap2 may play a crucial role in early development processes. Our current observations through knockout of *leap2* reveal the significant role of endogenous LEAP2 in regulating growth hormone expression and promoting somatic growth during zebrafish embryonic development.

Ethics declarations

Funding

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Ethical approval

All trials were supported by the Institutional Animal Care and Use Committee of Wenzhou Medical University.

Data availability statement

The data associated with my research have not yet been deposited into a publicly available repository. As a follow-up to "Data and Code Availability", the data may be made available on request.

CRediT authorship contribution statement

Kaiyu Guan: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Minjie Ye:** Resources, Methodology, Formal analysis. **Anqi Guo:** Writing – original draft, Methodology, Investigation. **Xiaoyu Chen:** Investigation. **Yunfeng Shan:** Writing – review & editing, Supervision, Project administration, Methodology. **Xi Li:** Writing – review & editing, Supervision, Project administration, Conceptualization.

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.

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