Piezo Type Mechanosensitive Ion Channel Component 1 is Required for Heart Development in Zebrafish

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Research Article

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Abstract

PIEZO1 is a non-selective cation channel protein that converts mechanical stimuli into electrochemical signals through mechanical force transmission. In recent years, more and more attention has been paid to its relationship with cardiovascular development and related diseases. However, PIEZO1 is difficult to be used as a therapeutic target due to incomplete study of its related phenotype and mechanism. Starting with the phenotypes of zebrafish at different stages of development, *piezo1* knockout zebrafish features decreased heart rate in embryonic stage, increased heart size in 72dpf larvae, as well as ventricular enlargement, passivation and increased immune infiltration in adult stage. Further characterization revealed the relationship between PIEZO1 and AcanA protein at embryonic stage, and hand2 protein at adult stage. Through further RNA-seq analysis, *ptpn21* downregulation, *shox2* upregulation, *itga4* upregulation were used to explain ventricular enlargement, heart rate decrease and immune infiltration increase respectively. Overall, this study provides a theoretical basis for elucidating the mechanism of PIEZO1 in regulating cardiac development.

Introduction

Cardiovascular disease becomes increasingly important as it contributes to high morbidity and mortality among all ages, especially for older adults. Despite the heavy burden it poses to the society, yet there is nothing like an optimal target for the disease because of its complicated mechanism[1].

The mechanically-activated (MA) cation channel protein encoded by Piezofamily is regulated by mechanotransduction. The Piezo family proteins, including PIEZO1 and PIEZO2, which participate in non-selective cationic channel activity, converse mechanical force into electrochemical signals[2].Unlike *Piezo2* which exists in aspects including mechanosensory neuron, damage sensing and respiration, *Piezo1* mainly distributes in non-sensory tissues, regulating osmotic homeostasis, vascular architecture, erythrocyte volume and epithelial cell division[3-7]. A wide range of researches show that PIEZO1 ion channel had important functions and its mutation related to human diseases, thus making it a considerable candidate for potential drug target[8-11]. In cardiovascular physiology, PIEZO1 is mainly supposed to be activated by the shear stress from increased blood flow or cell membrane stretch from increased blood pressure, which has close relationship with cardiovascular development and reconstitution after damage[12].

Research has shown that during early embryonic development, PIEZO1 plays an important role in vascular development, endothelial cell rearrangement and blood flow regulation[3, 12]. *Piezo1* channels are determinants of vascular structure in both development and adult physiology. Global or endothelial-specific disruption of mouse Piezo1 profoundly disturbed the developing vasculature and was embryonic lethal within days of the heart beating [3]. Studies in zebrafish have shown that under hypertension stimulation, PIEZO1 was involved in arterial remodeling process, and blocking PIEZO1-mediated signal transduction resulted in defective outflow tract and valve aortic development[13].*Piezo1* controls Klf2 and Notch activity in the endothelium and Yap1 localization in the smooth muscle progenitors to coordinate

OFT valve morphogenesis[14]. Studies in mice in which Piezo1 was conditionally deleted in endothelial cells demonstrated the requisite role of sphingosine 1-phosphate-dependent activation of Piezo1 in mediating angiogenesis *in vivo*[15]. Periodic stretching will induce cardiomyocyte rearrangement and PIEZO1 redistribution, and it also promote the growth of the aggregated cells expressing PIEZO1 in 3D hydrogel. Given the mechanical stimulation upon PIEZO1 inhibited, it can reduce eNOSlevel[16].

Among these current studies, although the role of PIEZO1 in the regulation of heart structure and function has been revealed, the deep mechanism of how PIEZO1 ion channel works remains unknown. There are more phenotype studies need to be carried on providing theoretical evidence for PIEZO1 as a therapeutic target. Therefore, in this study, we conduct research on *piezo1* KO zebrafish about its phenotype and cardiac function over different developmental stages. Additionally, we intend to figure out PIEZO1-regulated downstream genes specifically regarding cardiac functions and development, hoping to provide preliminary relationships with those phenotypes.

Materials And Methods

Zebrafish line:

The CRISPR/Cas9-mediated *piezo1*$^{+/-}$ zebrafish line in our experiments was a gift from Professor Jiu-linDu[17]. The single guide RNA (sgRNA) targeting the sequence of the zebrafish *piezo1* was designed at the exon 19, with the resulting mutation identified with a 2bp deletion in the exon 19, which leads to frameshift and premature stop (in the exon 20) of *piezo1.*

Zebrafish husbandry

The *piezo1*$^{+/-}$ zebrafish line and the wildtype zebrafish line were kept and raised in 10L water tanks in HaiSheng aquarium (ShanghaiTech University) systems, the tanks were maintained at 26°C with standard photoperiod. Male and Female *piezo1*$^{+/-}$ zebrafishes were mated to create a mixture of *piezo1*$^{+/+}$, *piezo1*$^{+/-}$, *piezo1*$^{-/-}$ zebrafish embryos. Embryos and larvae were raised in 1xE3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 10–5% Methylene Blue) until 8-10dpf, the fishes were transferred to the aquarium system. DNA extracts from tail fins were used to identify the genotypes of WT, *piezo1*$^{+/-}$, *piezo1*$^{-/-}$ adult zebrafish, mutations of *piezo1* were examined by PCR followed by sequencing analysis with primers (Forward: 5’- ATAATATTTAAGCTGATTCATAAGCTGCAGT-3’, Reverse: 5’-CAGCACATGTCTTTGGACTGTGGGGGAAA-3’). Fish experiments were

compliant to the general animal welfare guidelines and protocols approved by legally authorizedanimal welfare committees (ShanghaiTech University, ShanghaiTech Animal Welfare Committee).

Heart morphology and function characterization

Stereoscopic microscope was used to observe WT and *piezo1*$^{-/-}$zebrafish larvae 48hpf and 72hpf, the heart beats per minute of 72hpf zebrafishes were counted and recorded. The equipment was also used

for heart morphology observation and heart slice HE staining observation of 7–8-month-old WT, *piezo1*$^{+/-}$, *piezo1*$^{-/-}$ adult zebrafish. All figures of fish larva and heart slices were observed and shot by stereoscopic microscope.

Western Blotting:

The resected hearts of WT or *piezo1*$^{-/-}$adult zebrafish which were frozen with liquid nitrogen were lysed in RIPA (89900, Thermo Fisher) solution containing protease inhibitor cocktail (5892970001, Sigma). 4-20% ExpressPlus™ PAGE Gel (M42010C, GenScript) were used for protein samples separations. Samples were transferred onto PVDF membranes (FFP26, Beyotime) at 0℃, 200mA for 5h. Transferred membranes were blocked with 5% non-fat milk (A600669-0250, BBI) TBST (T1081, Solarbio) solution. The following primary antibodies were used: Piezo1 (1:500, 15939-1-AP ProteinTech) and GAPDH (1:500, D110016-0025, BBI). We used the following secondary antibodies: HRP-conjugated anti-rabbit secondary antibodies (1:5000, 65-6120,invitrogen) and anti-mouse secondary antibodies (1:5000, D110058-0025, BBI). HRP-conjugated antibodies were detected with chemiluminescence (ECL) detection kit (ED0015-C, SparkJade).

Quantitative Real time PCR (RT-qPCR):

The total RNA from resected heart in adult or larval zebrafish was extracted with TRIzol Reagent (235002, Ambion), followed by separation with chloroform and purification with isopropanol and 70% alcohol. cDNA was synthesizedAwithPrimeScript RT Master Mix (RR036A-1, Takara) and cDNA samples were amplified with *shox2*, *cenpf*, *itga4*, *dsc2l*, *tfpi2, smyd1b*, *uqcrb*, *fbxw7* and*ptpn21* with ChamQ Universal SYBR qPCR Master Mix (Q711-02, Vazyme). Reactions were run and analyzed with *gapdh* as an endogenous internal control. Primers used are listed in supplementary table 1. Relative expression values for each gene were calculated using the ΔΔCt analysis method.

H&E staining:

The hearts excised from ether WT or *piezo1*$^{-/-}$ adult zebrafish were fixed in 10% neutral buffer formalin for at least 24h. After gradient dehydration in alcohol and infiltration in xylene, the excised tissues were embedded in paraffin and were sliced into 7-8mm sections. Then the deparaffinized tissues were histologically examined following hematoxylin (E607367-0100, BBI) and eosin (E607321-0100, BBI) staining.

RNA-Seq and data analysis:

RNA was isolated following standard TRIzol protocols. After RNA extraction, the sample was shipped for library preparation.Sequencing was performed using HiSeq2500(Illumina Inc., San Diego, CA) at Genergy Biotechnology (Shanghai) Co., Ltd. Pre-alignment quality control and trimming were performed by fastp[18], removing reads less than 35 bases in length and PHREAD less than 20. Remaining reads were mapped to *Danio Rerio* GRCz11 reference genome using sensitive-local mode in bowtie2

aligner[19].edgeR[20] was used to detect DE genes between control and Piezo1$^{-/-}$ groups. Genes were removed when they had a total read count of zero. Common dispersion was estimated by housekeeping genes from GDS3623 (DPM<0.01) as shown in supplementary table 2. Pairwise comparison between two groups showed differentially expressed genes (DEGs). *P*values were adjusted following Benjamini& Hochberg method, controlling the false discovery rate (FDR). Genes were regarded to be DEGs if the FDR was below 0.05 and fold change greater than 2.

Statistical analysis:

Results were expressed as mean ± standard deviation (SD). Mean differences between two groups were analyzed by Student's two-tailed t-test and comparisons of multiple groups were analyzed by one-way ANOVA (GraphPad; Prism) *P* values < 0.05 were considered significant. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

Result

3.1 Identification of PIEZO1 expression level in *piezo1* KO zebrafish

As the basis of following phenotypic experiment, we first confirmed the decrease of PIEZO1 expression level at both transcription and translation levels in homozygous *piezo1* KO zebrafish. Furthermore, the data in transcription level from wild, heterozygous and homozygous KO strains showed that *piezo1* gene has a certain quantitative effect.

3.2 Phenotype identification of *piezo1* KO zebrafish in different development level

To compare the phenotypic changes of *piezo1* KO homozygous zebrafish heart at different developmental stages, 48hpf and 72hpf fish larvae and adult fish at 7-8 month were selected for phenotypic analysis. From the perspective of morphology analysis, no sign of clear heart development abnormity was identified in fish larvae at 48hpf though certain individual differences were observed (Figure 4a). However, the heart of 72hpf fish larvae showed increased heart size and volume, shown through both the diameter and the depth of the developing heart, and with certain degrees hydropericardium. Then, we characterized 72hpf juvenile fish at which stage clear heartbeat can be observed and found that the heart rate of the homozygous mutants experienced a major decline (Figure 4c). Decrease of blood-pumping ability of the heart was also observed (Data not shown). The adult mutants experienced phenotypic transformation of ventricular enlargement, shown through the passivated ventricular contour and lack of apparent apex (Fig 4d), which was reminiscent of the similarly enlarged heart at 72hpf. This morphology change was also confirmed through HE staining. Additionally, analysis of cell species showed an increase in immune cell infiltration among mutant cardiac cells (Fig 3g-3i).

3.3 Expression level of heart development key genes in *piezo1* KO zebrafish at different development stages

In this study, we chose genes related to the development and migration of embryonic central myocardial cell (*grinch, s1p2, sdc2*), genes related to the differentiation of myocardial cell (*gata5, hand2*), genes related to the development of artery (*ace/gfg8*), vein (*Islet-1*), arterial glands (*elastin b*), aortic valve (*Notch1*), atrioventricular valve(*Ugdh*), vascular endothelial cell (*Fn1, klf2a*), ventricular myosin (*vmhc*) and extracellular matrix of cardiac cells (*has2, AcanA, elastin b*)[21]. All the genes were detected in wildtype zebrafish and Piezo1 KO homozygous zebrafish at different stages of development. In 48-hour-embryo, the expression level of mutant *AcanA* Genes showed an obvious up-regulation (Fig 4a). However, the expression of level of these gene did not show any significant changes in whole adult zebrafish lysates (Supplementary Fig 1), but the expression level of *hand2* gene showed a significant decrease in dissected hearts of adult zebrafish. Since *hand2* is a key factor involved in the differentiation of cardiac muscle cells, this expression will further investigate the changes in the expression level of potential upstream pathway of *hand2*. Previous research has shown that *nkx2.5* plays an important role in the signal transduction in cardiac cells, as the upstream or parallel factor of *hand2*[21]. Additionally, studies have shown that Hippo pathway can regulate the expression of BMP and *hand2* signals through LATS1/2, thus influencing the differentiation of cardiac cells[22]. These two possible upstream pathways were selected for second sound of expression level examination, but no significant transcriptional changes were found (Fig 4c,4d).

3.4 Identification of differentially expressed genes in hearts of *piezo1*$^{-/-}$ zebrafish

To study transcriptomic changes, *piezo1*$^{-/-}$ heart RNA sequencing was conducted. It was indicated that *piezo1* knockout leads to 818 differentially expressed genes (DEGs), in which 535 were upregulated (Fig. 5a). Among these, we concentrated on several DEGs that are related to heart functions, namely upregulated *shox2*, *dsc2l*, *hbegfb*, *tfpi2* and downregulated *smyd1b*. Combined with GO annotations, 5 more genes that were not regarded as DGEs but related to heart functions were selected out (Fig. 5b), including *itga4*, *ptpn21*, *cenpf*, *uqcrb* and *fbxw7*. Altogether, the expression changes of these 10 genes were examined through qPCR (Fig. 5c) and showed consistent expression changes in RNA-seq data: *shox2*, *cenpf*, *itga4*, *dsc2l* and *tfpi2* were upregulated, whereas *smyd1b*, *uqcrb*, *fbxw7* and *ptpn21* were downregulated. These heart-function-related genes mainly involve in 3 pathways: heart development enriched by *shox2*, *tfpi2*, *dsc2l*, *cenpf*, *ptpn21* and *smyd1b*, heart contraction enriched by *shox2*, *smyd1b* and *hbegfb*, and vasculature development enriched by *itga4*, *fbxw7*, *uqcrb* and *cenpf*.

GO enrichment analysis showed top terms (Fig. 5d, top) in biological process (phosphatidylcholine-sterol O-acyltransferase activator activity, cholesterol binding, and cholesterol transporter activity), cellular component (nucleosome, extracellular space and extracellular region) and molecular function. To further explore the potential pathways DGEs participated in, GO enrichment analysis revealed that DGEs were also closely related to immune responses such as defense response against bacterium, innate immune response and inflammatory response, as well as cholesterol or lipid metabolism including HDL particle assembly, positive regulation of cholesterol esterification and VLDL particle remodeling (Fig. 5d, down).

Discussion

Piezo1 knockout caused three distinct phenotypes including reduced heart rates in 72dpf zebrafish larvae, heart/ventricular enlargement in 72hpf and adult zebrafish, and immune infiltration in adult zebrafish, suggesting defects in heart development and subsequent cardiac function.

Regarding ventricular enlargement, the downregulation of *ptpn21* seemed to be relevant. Since *ptpn21* morphant caused severe pericardial edema [23] in zebrafish embryo, it is possible that downregulated *ptpn21* contributes to hydropericardium as well as ventricular enlargement as a compensatory affect.To notice, there is no direct evidence reporting on the effect of long-lasting disturbance of *ptpn21* and therefore the mechanism through which ventricular enlargement was caused may be novel.

For reduced heart rate, we argued that disrupted *shox2* expression may lead to developmental defects, as previous research showed the relationship between *shox2* mutant and bradycardic arrhythmia, one of whose symptoms is reduced heart rate[24]. Again, the assumption made behind this hypothesis was that the abnormal function was due to developmental failure, as the short stature homeobox 2 (*shox2*) transcription factor is crucial in the formation and differentiation of the sinoatrial node (SAN) [24].

Surprisingly, although PIEZO1 was reported to be essential for innate immunity, the effect of PIEZO1 on immunity in heart remains unclear. One possible explanation for the immune infiltration observed could be upregulation of *itga4*, which triggers homotypic aggregation for most VLA-4-positive leukocyte cell lines [25], and therefore prompts inflammatory responses.

Here, we reviewed some of the most noticeable DE genes carried out by RNA-seq. According to functional analysis, these genes were divided into three classes - heart development, heart contraction and vasculature development. Several DE genes were closely related to heart development. The late mitotic protein centromere protein F (cenpf) known to interact with microtubules was reported to contribute to all hallmarks of dilated cardiomyopathy (DCM) in a loss-of-function model in cardiac myocytes[26]. Previously reported depletion of tissue factor pathway inhibitor-2 (*tfpi2*) in zebrafish embryos caused retrenched ventricle, enlarged atrium, malformation of atrioventricular boundary and disrupted sarcomere organization [27]. *dsc2l* was inferred to be an orthologue to human desmosomal cadherin desmocollin-2 [28], which was suggested as conferring susceptibility to inheritable DCM by human genetic studies [29]. The SET- and MYND domain containing protein 1b (*smyd1b*) is a member of the Smyd family that is specifically expressed in skeletal and cardiac muscles. *smyd1b* plays a key role in thick filament assembly during myofibrillogenesis in skeletal muscles of zebrafish embryos, knockdown of *smyd1b* causes significant disruption of myofibril organization in both skeletal and cardiac muscles of zebrafish embryos[30].

As for heart contraction, heparin-binding epidermal growth factor (EGF)-like growth factor (HBEGF) is a ligand for the EGF receptor (EGFR). Functional assessment in zebrafish demonstrates that when HBEGF expression is reduced with morpholino antisense reagents, it can result in a phenotype of myocardial contractile dysfunction [31]. It was reported that zebrafish Integrin alpha4 (*itga4*) and alpha5 function redundantly in cardiac morphogenesis and endocardial differentiation [32].

Lastly, we look at genes related to vasculature development. UQCRB loss of function by either genetic and pharmacological means inhibited angiogenesis [33]. F-box and WD repeat domain–containing 7 (FBXW7) is an E3-ubiquitin ligase, which serves as one of the components of the SKP1, CUL1, and F-box protein type ubiquitin ligase (SCF) complex. Previous study identified FBWX7 as an important regulator of cardiac hypertrophy [34].

Taken together, this article favors further mechanistic study on the effect of Piezo1 knockout in zebrafish.

Declarations

Conflict of interest All authors declare that they have no conflict to interest.

Consent to participate Written consent was obtained from all authors. This study was performed in strict accordance with guidelines for the care and use of laboratory animals for ShanghaiTech University. The protocols were approved by the the Shanghaitech Ethical Use of Aminals Committee (20200903003) All procedures using zebrafish were performed under Tricane anesthesia, and every effort was made to minimize discomfort and suffering.

Consent to publish All authors approved the current state of this manuscript.

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Yufeng Liu and Zhiyu Fang have contributed equally to this work.

Author contributions LY, CY, SJ maintained the zebrafishes and performed the experiments. FZ analyzed the RNA-seq data. ZS participated in the western blot. KYJ and SJL supervised the study. All the authors wrote the manuscript.

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Figures

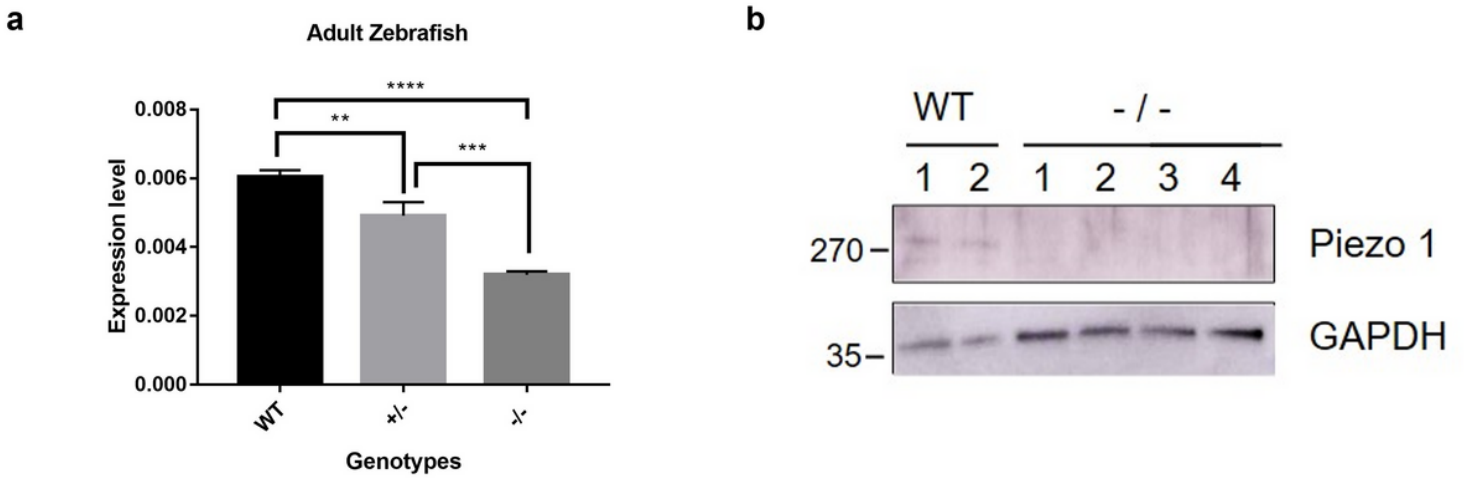

Figure 1

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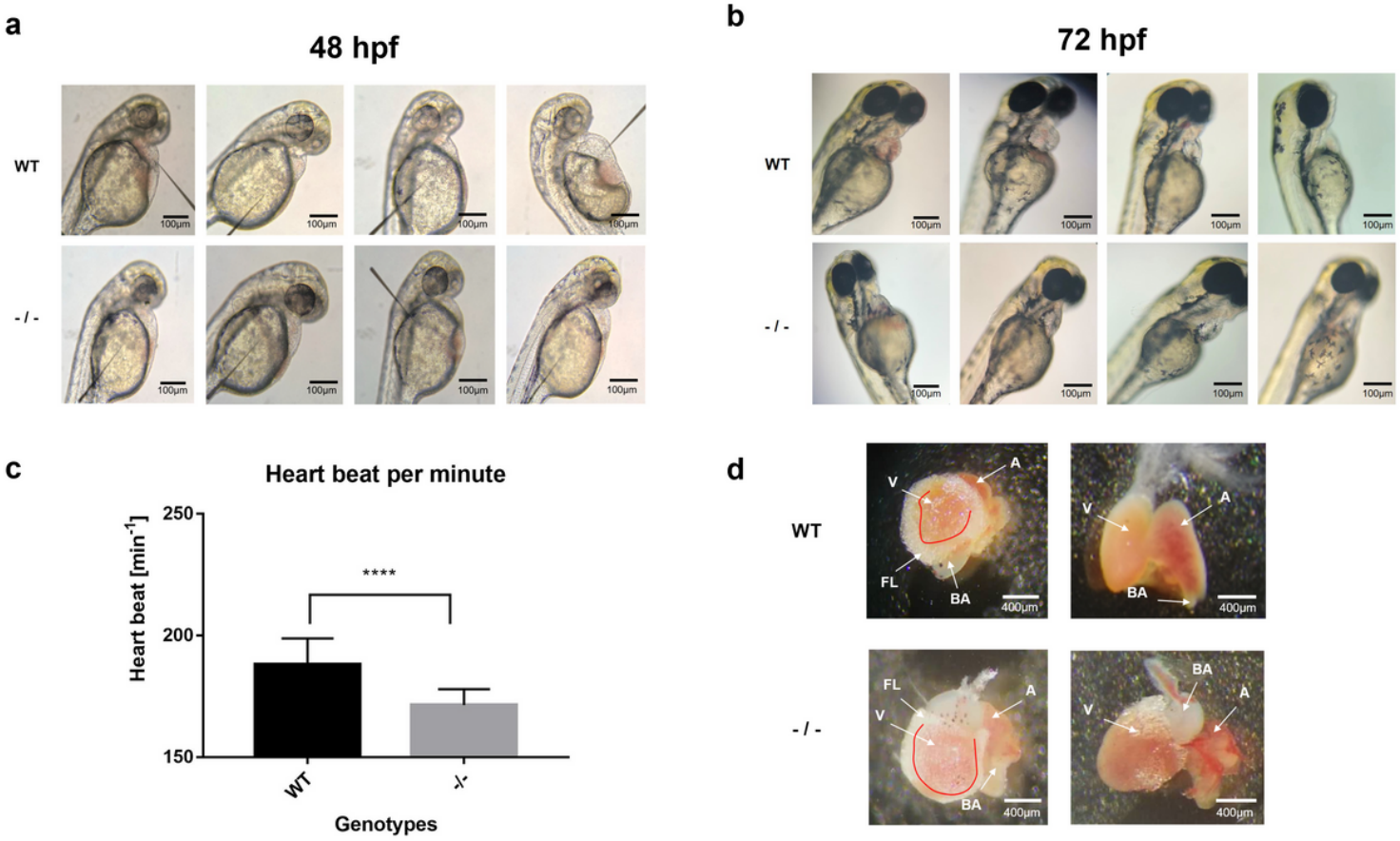

Figure 2

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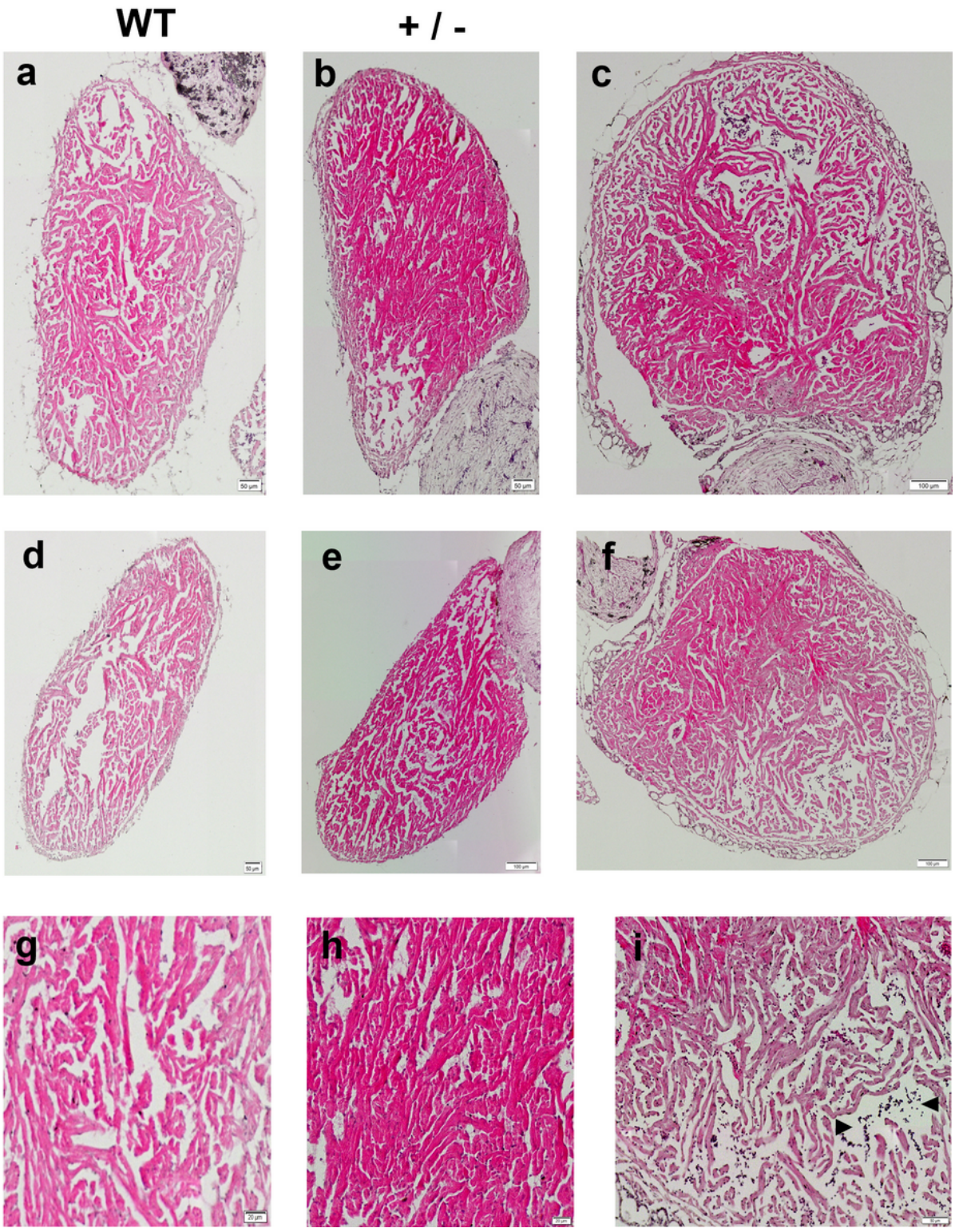

Figure 3

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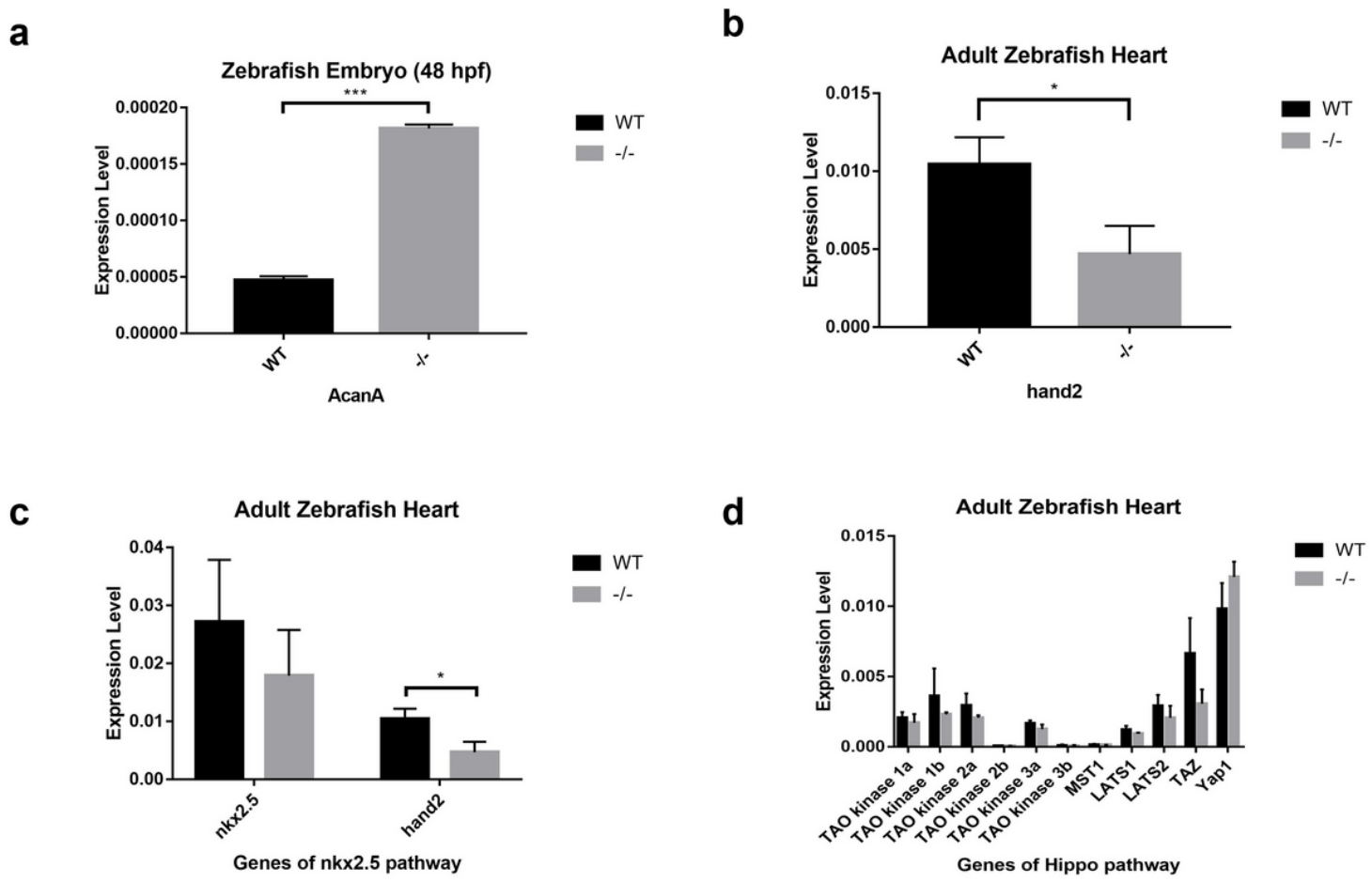

Figure 4

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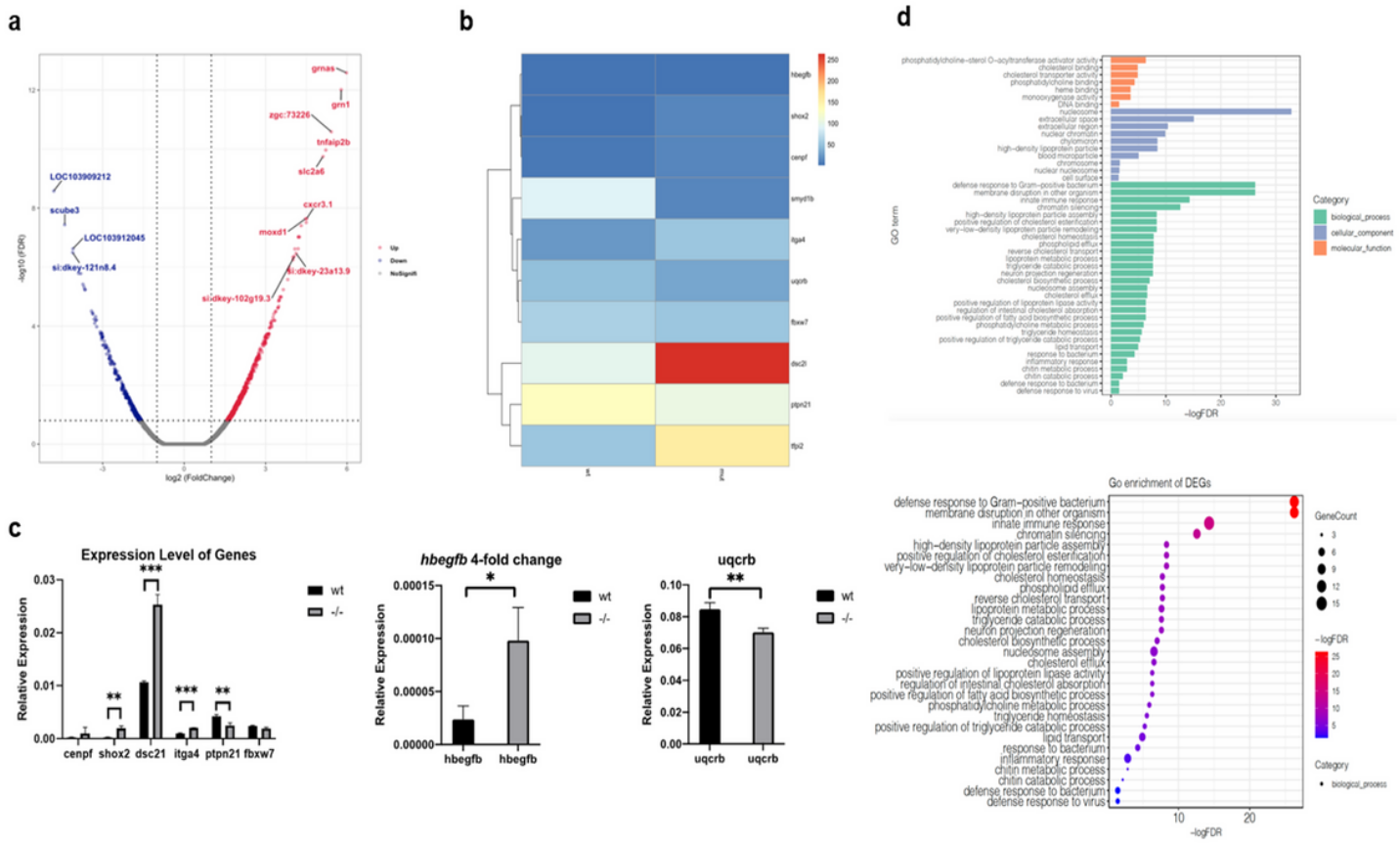

Figure 5

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