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团头鲂*lpin1*基因的克隆、表达及对肌肉损伤的响应

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摘要 为深入研究*lpin1*基因在鱼类肌肉损伤修复过程中的作用机制,以团头鲂(*Megalobrama amblycephala*)为研究对象,采用荧光定量PCR和整胚原位杂交技术检测团头鲂*lpin1*基因的时空表达及肌肉损伤后该基因的表达变化。结果显示,团头鲂*lpin1*基因的ORF序列长2 694 bp,编码897个氨基酸,编码的蛋白质具有高度保守的Lipin-N、Lipin-mid和LNS2(Lipin/Ned1/Smp2)结构域,系统进化树分析显示团头鲂Lpin1与鲤和斑马鱼的亲缘关系最近。荧光定量PCR结果显示,*lpin1*在团头鲂胚胎发育过程中的各个时期均有表达,且在成鱼肌肉组织中表达量最高;整胚原位杂交进一步显示*lpin1*在肌肉效应期和心跳期的头部和体节中特异表达;对团头鲂幼鱼肌肉进行不同程度损伤后,与未损伤组相比,*lpin1*基因分别在轻度损伤后48 h和重度损伤后72 h的表达量最高,*pax7*基因表达量分别在轻度损伤后144 h和重度损伤后96 h最高。以上结果表明,*lpin1*基因可能在团头鲂肌肉损伤和修复的过程中扮演着重要角色。

关键词 团头鲂; *lpin1*基因; 基因表达; 肌肉损伤修复

中图分类号 Q959.46⁺⁸ **文献标识码** A **文章编号** 1000-2421(2024)05-0205-08

骨骼肌卫星细胞(skeletal muscle satellite cell, MuSC)是参与肌肉受损后修复和再生过程的肌肉干细胞^[1]。一旦肌肉受到损伤,MuSC就会响应并退出静止状态,然后增殖成为生肌前体细胞或肌母细胞^[2],增殖结束后分化成肌细胞,以相互融合或和肌纤维融合的方式来修复受到损伤的肌肉组织,并补充骨骼肌卫星细胞数量^[3-4]。在这个过程中,MuSC会受到多种因子的调控,特别是以Pax7(paired box 7)表达为特征的MuSC发挥着十分关键的作用,缺乏Pax7的卫星细胞,会导致肌肉再生失败^[5-6]。

Lpin1既可以作为一种磷脂酸磷酸化酶(phosphatidic acid phosphohydrolase, PAP)参与磷脂和甘油三酯的合成^[7],也可以作为一种转录辅激活因子,调节脂类代谢相关基因的表达^[8]。之前的研究发现Lpin1在脂肪分解和脂肪细胞分化^[9-10]、肝脏脂肪酸和脂蛋白生成^[11]以及线粒体自噬中都发挥重要作用^[12-14]。LPIN1缺乏症患者会出现肌肉萎缩和横纹肌溶解症^[15-16],LPIN1基因杂合子突变的患者也易患他汀类药物诱导的肌病^[17]。LPIN1缺乏的脂肪肝

营养不良小鼠在肌肉损伤后,再生肌纤维横截面会减少,肌分化因子(myogenic differentiation, MyoD)的表达降低^[18]。另外,小鼠Lpin1基因缺乏会导致其骨骼肌纤维表型显著变化,肌膜下线粒体质量也有所增加^[19]。在斑马鱼中,*lpin1*基因的缺失会导致肌分裂缺陷,初级和次级运动神经元减少^[20]。以上研究说明,磷脂酸磷酸化酶基因可能在骨骼肌损伤后修复的过程中扮演着重要角色。

前期基于团头鲂骨骼肌损伤后的转录组数据分析发现,*lpin1*基因在团头鲂肌肉损伤后差异表达^[21],为进一步研究该基因的功能,本研究克隆团头鲂*lpin1*基因,检测*lpin1*在团头鲂成鱼各组织及胚胎发育过程中的表达模式,并分析肌肉损伤对该基因表达的影响,旨在为深入研究*lpin1*基因在鱼类肌肉损伤修复过程中的作用机制提供理论依据。

1 材料与方法

1.1 肌肉损伤试验和样品收集

本研究使用的团头鲂幼鱼(1龄)全长(15.62±

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2.33) cm, 购自黄冈团头鲂养殖场, 充氧袋打包运回华中农业大学水产学院试验基地, 并于池中暂养2周后用于肌肉损伤试验。供试鱼用0.1%的MS-222(Sigma)麻醉后, 用直径0.2 cm的针头插入其排泄孔上方侧线附近的肌节中进行肌肉损伤操作, 其中轻度损伤的长度约为0.3 cm, 重度损伤的长度约为1 cm, 深度约为0.5 cm。将损伤后的团头鲂鱼体消毒后立即放入清水中, 正常养殖。分别于损伤后0、24、48、72、96、144、216 h取损伤部位的肌肉组织(规格为1.0 cm×1.0 cm), 所取样品立即放入液氮中保存, 每个时间点取9尾鱼样本。

试验所需胚胎及仔鱼是通过3对团头鲂亲本人工授精而来。受精卵在平均水温27℃左右的孵化桶中进行孵化, 解剖镜下观察胚胎发育情况, 取样后立即放入液氮中保存。原位杂交所用胚胎去卵膜后于

4%多聚甲醛中4℃固定24 h后脱水至甲醇中-20℃保存。另外, 采集团头鲂成鱼($n=3$)肝脏、鳃、心脏、大脑、肠道和肌肉等6个组织样品, 用于分析目的基因在各组织中的表达情况。

1.2 总RNA提取及cDNA合成

参照TRIzol® Reagent说明书提取各时间点团头鲂胚胎、仔鱼和成鱼各组织及损伤肌肉的总RNA, 用HiScript® II QRT SuperMix for qPCR(+gDNA wiper)试剂盒将提取的RNA反转录成cDNA后保存至-20℃。

1.3 基因的克隆

从笔者所在实验室前期已有的团头鲂转录组数据中获得*lpin1*基因的序列, 通过PCR验证该基因的ORF序列, 并送至武汉天一辉远科技有限公司测序, PCR引物见表1。

表1 所用引物序列

Table 1 Primer sequences used in this study

引物名称 Primer name	引物序列 Primer sequence (5'-3')	用途 Application
lpin1-ORF-F	ATGAACCTACGTGGGTCAGTTGGC	扩增ORF
lpin1-ORF-R	TTAGCTGCTCTGAGCATGTTGATCC	Amplifying ORF
lpin1-WISH-F	TACGCACCTCAGAGCCATCAC	合成探针
lpin1-WISH-R	TAATACGACTCACTATAGGGGACGCTGAAGACCACATC	Synthesizing probes
lpin1-qRT-F	ACCCCTCTCCGGCTGTATTGA	
lpin1-qRT-R	CTGACCGGCTCCCCATTAT	
pax7-qRT-F	GAGGCCTCTCCGTTAGCTC	荧光定量PCR
pax7-qRT-R	GCTGCGTCTCTGTTCCCTCT	Fluorescent quantitative PCR
ef-1 α -qRT-F	CTTCTCAGGCTGACTGTGC	
ef-1 α -qRT-R	CCGCTAGCATTACCCCTCC	

1.4 生物信息学分析

运用在线网站(www.ncbi.nlm.nih.gov/orffinder)预测氨基酸序列; 使用MAGA 6.0软件构建Lpin1蛋白的系统进化树、DNAMAN软件进行Lpin1蛋白的多序列比对; 蛋白质信号肽预测、蛋白质的理论分子质量和理论等电点分析分别用软件SignalP([www.cbs.dtu.dk/services/SignalP/](http://cbs.dtu.dk/services/SignalP/))和ProtParam(<http://web.expasy.org/protparam>)进行; 利用NCBI的CDD数据库(www.ncbi.nlm.nih.gov/cdd)预测Lpin1蛋白的结构域。

1.5 荧光定量PCR

为分析*lpin1*基因的时空表达谱, 本研究分别以团头鲂成鱼各组织、胚胎发育各时期和不同损伤程度的肌肉的cDNA为模板, 以 $ef-1\alpha$ 作为内参基因, 试验步骤参照文献[22], 结果通过 $2^{-\Delta\Delta Ct}$ 法计算目的基因的相对表达量并使用GraphPad Prism 6进行制图。

数据以Mean±SD表示, 采用单因素方差分析方法(ANOVA)和Duncan's进行差异表达分析, 当 $P<0.05$ 时为差异显著。

1.6 整胚原位杂交

以团头鲂cDNA作为模板扩增*lpin1*基因, PCR产物回收后参照下列反应体系进行体外转录: 回收产物1 μg, DIG-labeling UTP Mix(10×)1 μL, Transcription buffer(5×)2 μL, T7/SP6 RNA聚合酶(20 U/μL)1 μL, RNase Inhibitor 1 μL, DEPC水加至20 μL; 吹打混匀后37℃水浴2 h, 然后加入1 μL DNaseI 37℃水浴15 min除去DNA后合成探针。整胚原位杂交的步骤参照文献[23]进行。

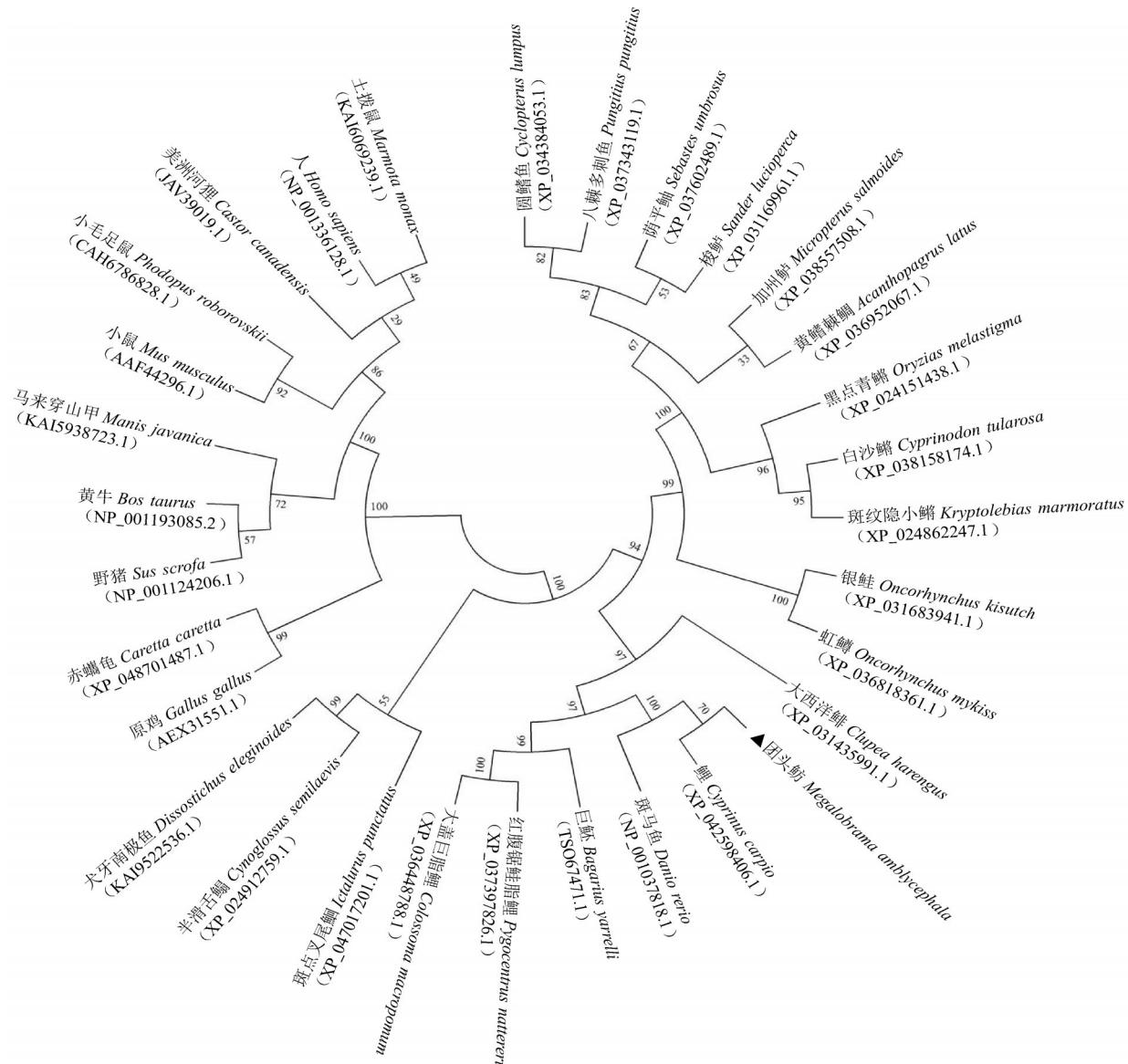
2 结果与分析

2.1 基因的序列分析

团头鲂*lpin1*基因的ORF序列长度为2 694 bp,

编码 897 个氨基酸。预测的蛋白理论分子质量为 98.93 ku, 理论等电点为 5.43。SignalP 预测结果显示, *Lpin1* 蛋白 N-末端无信号肽, 为非分泌型蛋白。基于多个物种 *Lpin1* 氨基酸序列的系统进化分析显示, 鱼类聚为 1 个分支, 爬行类、鸟类和哺乳类形成另一分支, 在鱼类分支中团头鲂与鲤和斑马鱼的亲缘

关系最近(图 1)。多序列比对结果如图 2 所示, 团头鲂 *Lpin1* 和鲤的相似性最高(80.92%), 其次是斑马鱼(79.55%), 最低的是白沙鱥(53.33%)。对不同物种 *Lpin1* 蛋白结构域分析表明, 该蛋白的氨基端、中间和羧基端都有一段高度保守的序列, 分别为 *Lipin-N*、*Lipin-mid* 和 *LNS2*(*Lipin/Ned1/Smp2*)结构域。



带三角形的为团头鲂 *Lpin1* 蛋白, 节点上的数值表示 bootstrap。*Lpin1* protein of *M. amblycephala* was shown in black triangles, value on the nodes represent the bootstrap.

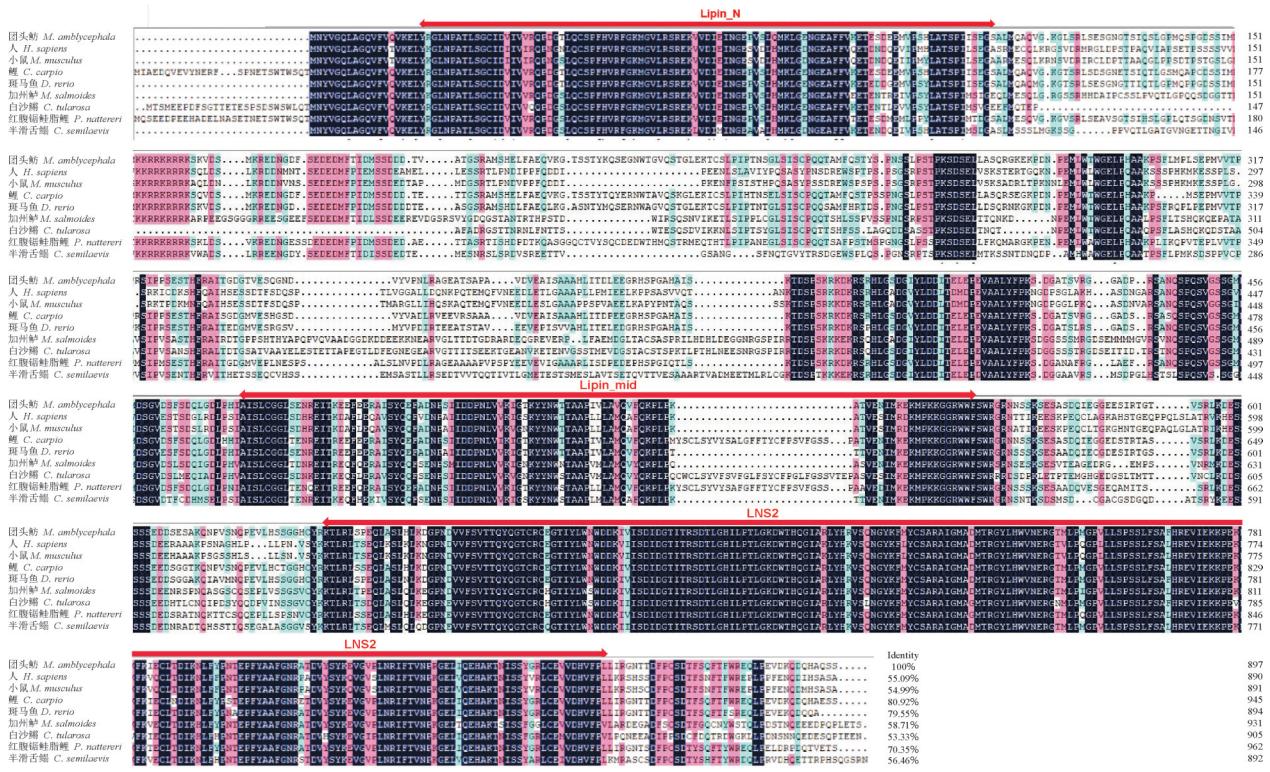
图 1 *Lpin1* 的系统进化分析

Fig.1 Phylogenetic analysis of *Lpin1*

2.2 团头鲂 *lpin1* 的时空表达

使用荧光定量 PCR 分析 *lpin1* 基因在团头鲂成鱼各组织和胚胎各发育阶段的表达, 结果发现 *lpin1* mRNA 在肌肉和心脏中的表达量最高且显著高于其他组织(图 3)。另外, *lpin1* 基因在胚胎发育过程中的

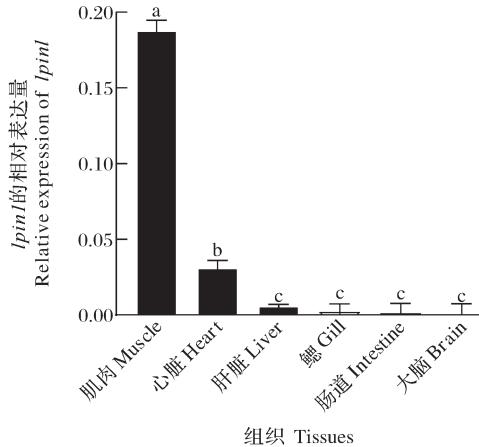
各个时期均有表达, 但在出膜后的表达量相对较高, 且随着发育的进行其表达量逐渐增加并在出膜后 8 d 达到峰值(图 4)。整胚原位杂交结果进一步显示, *lpin1* 在受精卵期就能检测到高表达, 并且在体节生成期前的各时期呈现持续而一致的泛表达模式。从



Lipin-N、Lipin-mid 和 LNS2 分别为 3 个保守的结构域。Three conserved domains, Lipin-N, Lipin-mid and LNS2 were found.

图 2 Lpin1 蛋白的氨基酸序列多重比对

Fig. 2 Multiple alignment and analysis of amino acid sequences of Lpin1 proteins



柱上不同字母表示差异显著, $P < 0.05$ 。下同。Different letters indicated significant difference ($P < 0.05$). The same as below.

图 3 lpin1 基因在团头鲂成鱼各组织中的相对表达量

Fig. 3 The relative expression of lpin1 in adult

M. amblycephala tissues

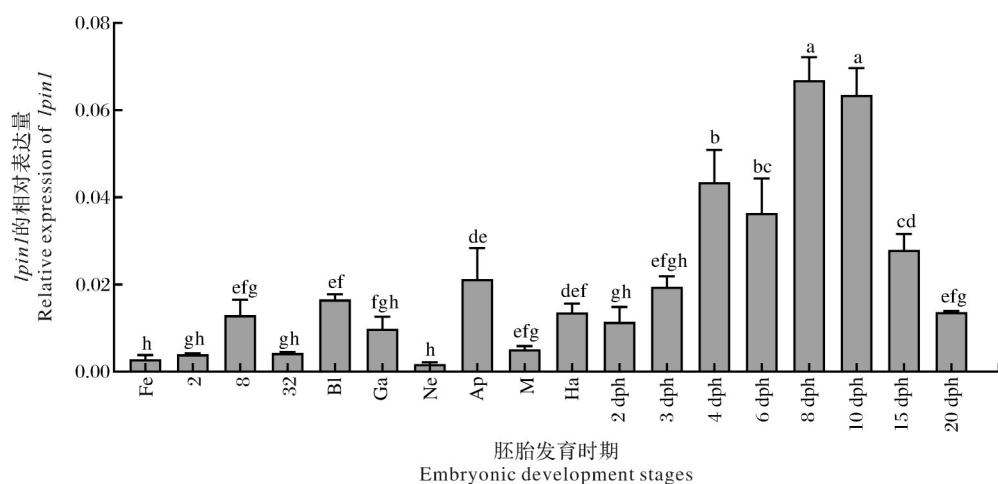
肌肉效应期开始,可以在体节肌中观察到弱表达,而在眼、端脑、中脑以及后脑中检测到强信号,心跳期表现得尤其明显;随后的出膜期,其表达主要在大脑、肝脏和肠道,并且在体节肌中持续表达(图 5)。

2.3 肌肉损伤后团头鲂 *lpin1* 和 *pax7* 基因表达的变化

荧光定量 PCR 结果显示,与未损伤组相比, *lpin1* 和 *pax7* 基因在轻度和重度损伤后 24 h 的表达均下调。轻度损伤后, *lpin1* 表达量在 48 h 达到峰值,然后降低,但在 48~216 h 其表达量均高于对照组;而 *pax7* 基因表达量在损伤后 96 h 显著升高,并在 144 h 达到最高。重度损伤后, *lpin1* 表达量在 72 h 和 216 h 时显著高于对照组; *pax7* 表达量在 48 h 和 96~216 h 均显著高于对照组,且在 96 h 达到峰值(图 6)。

3 讨 论

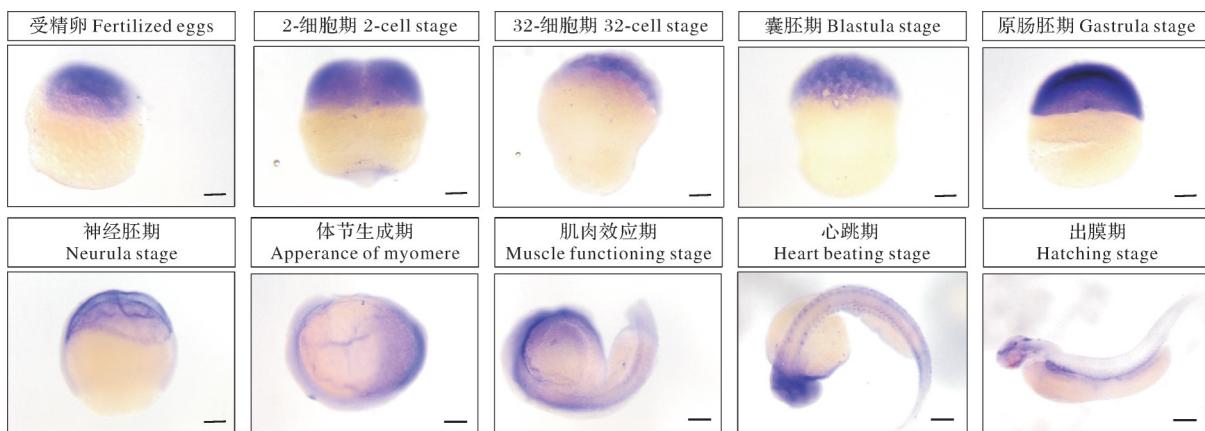
本研究获得了团头鲂 *lpin1* 基因 ORF 全长 2 694 bp, 编码 897 个氨基酸。通过对不同物种功能结构域分析发现,该蛋白的氨基末端和羧基末端都有一段高度保守的序列,分别为 Lipin-N 和 LNS2(Lipin/Ned1/Smp2)结构域。其中 LNS2 结构域包含了磷脂酸磷酸酶活性序列 DxDxT 和转录共激活因子功能的活性位点基序 LxxIL, DxDxT 的存在将 Lpin1 鉴定



Fe:受精卵时期;2:2-细胞期;Bl:囊胚期;Ga:原肠胚期;Ne:神经胚期;Ap:体节期;M:肌肉效应期;Ha:出膜期;dph:出膜后天数。
Fe: Fertilized eggs; 2: 2-cell stage; Bl: Blastula stage; Ga: Gastrula stage; Ne: Neurula stage; Ap: Appearance of myomere stage; M: Muscle functioning stage; Ha: Hatching stage; dph: Days post hatching.

图4 *lpin1*基因在团头鲂胚胎各发育阶段的相对表达量

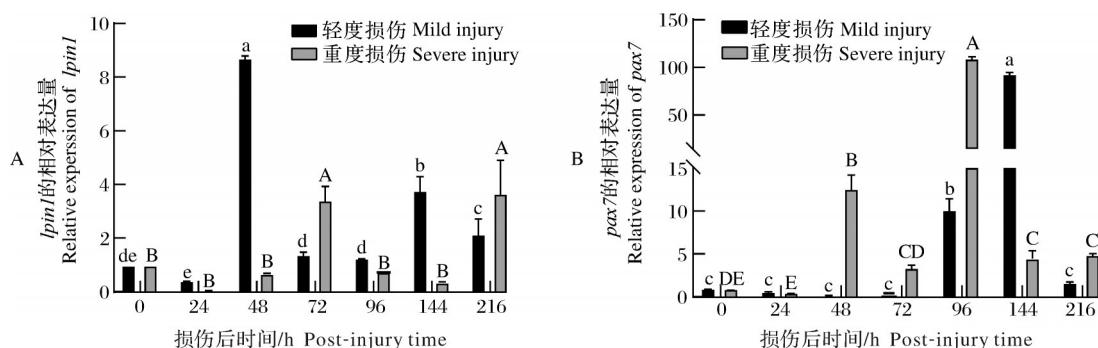
Fig.4 Relative expression of *lpin1* at *M. ambycephala* different embryo development stages



图中蓝色为杂交信号,标尺:200 μm。Blue for hybridization signals of *lpin1* gene. Scale bars: 200 μm.

图5 原位杂交技术检测团头鲂*lpin1*基因在胚胎不同发育时期的表达

Fig.5 Expression of *lpin1* gene at different embryo development stages of *M. ambycephala* based on *in situ* hybridization



不同小写(大写)字母表示不同时间轻度(重度)损伤之间有显著性差异, $P<0.05$ 。Different lowercase letters(uppercase letters) indicated significant difference between mild injuries (severe injuries) at different times, $P<0.05$.

图6 团头鲂*lpin1*(A)和*pax7*(B)基因在肌肉损伤修复过程中的表达变化

Fig.6 Expression changes of *lpin1* (A) and *pax7* (B) genes during muscle repair of *M. ambycephala*

为卤代酸脱卤酶超家族(haloacid dehalogenase superfamily, HAD)的成员^[24], PGC-1 α 和PPAR α 能通过与LxxIL直接作用调节脂肪酸氧化基因的转录^[8]。进化树分析结果显示团头鲂与其他鱼类聚为一个分支,且与鲤和斑马鱼的亲缘关系最近。Lpin1的结构域和进化树分析说明其在进化过程中可能具有保守的生物学功能。

本研究发现*lpin1* mRNA在团头鲂成鱼肌肉和心脏中的表达显著高于其他组织。类似地,在小鼠中,*lpin1*基因在脂肪、心脏和骨骼肌中高表达^[7, 25]。斑马鱼中,*lpin1*基因在胚胎早期发育过程中泛表达,随后开始在体节中特异表达,受精后24 h在头部和体节肌表达,受精后72 h仅在神经嵴、头肌和胸鳍处检测到,但受精后6 d在肌节特异性表达恢复^[20]。与在斑马鱼中研究相符,我们的研究结果显示,*lpin1* mRNA从受精卵到体节生成期呈泛表达;肌肉效应期和心跳期在眼和脑中检测到强表达,出膜期在脑、肝脏和肠道的特异表达尤为明显,在这个过程中,*lpin1*在体节肌中持续弱表达。

当骨骼肌出现损伤后,肌卫星细胞被激活,通过增殖、迁移和分化形成肌细胞,经过融合过程后形成新的肌纤维以修复受伤的肌肉组织^[7, 26-27]。肌卫星细胞增殖分化过程中,受到生长因子和转录因子等不同类型信号分子的调控^[28-29]。Pax7作为肌肉发育和再生所需的MuSC标志物^[30],在哺乳动物损伤后骨骼肌再生中发挥关键作用^[31-32]。研究发现团头鲂肌肉损伤后,Pax7 $^+$ 细胞的数量在损伤后96~144 h达到峰值,且重度损伤的肌肉中Pax7 $^+$ 细胞的增殖比轻度损伤的肌肉更显著^[21]。在斑马鱼中,*pax7*和*pax3*的表达水平在损伤后4~5 d达到峰值^[33]。在小鼠成肌细胞中,Lpin1可以抑制细胞外信号调节激酶1/2(extracellular signal-regulated kinase 1/2, ERK1/2)磷酸化进而抑制肌肉再生^[18],而ERK信号的激活发生在肌肉再生的早期阶段^[34]和伤口愈合过程中^[35]。另外,在脂肪肝营养不良中Lpin1缺乏会影响骨骼肌的再生能力^[18],斑马鱼中*lpin1*缺失也会导致骨骼肌发育受损^[20]。本研究也发现与损伤前相比,*lpin1*基因在肌肉损伤修复过程中的表达发生显著变化,这些结果都说明*lpin1*基因可能参与肌肉的损伤修复过程。

综上,本研究克隆获得团头鲂*lpin1*基因,其Lipin-N、Lipin-mid和LNS2结构域在进化上高度保守。时空表达结果显示*lpin1*在团头鲂成鱼肌肉中的表达

量最高,从受精卵到出膜后20 d的各个时期均有表达,且肌肉效应期和心跳期时在头部和体节中特异表达。另外,本研究也发现团头鲂肌肉不同程度损伤后,*lpin1*与*pax7*基因表达变化呈现相似趋势,这些结果可为研究*lpin1*基因在鱼类肌肉损伤和修复过程中的分子机制奠定基础。

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Cloning and expression of *lpin1* gene and its response to muscle injury in *Megalobrama amblycephala*

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Abstract To study the role of lipin1 (*lpin1*) gene in the repair of muscle injury in fish, taking *Megalobrama amblycephala* as the research object, in this study, the temporal and spatial expression of *lpin1* gene and its expression change after muscle injury were detected by fluorescent quantitative PCR (qPCR) and whole embryo *in situ* hybridization (WISH). The results showed that the ORF sequence of the *M. amblycephala lpin1* gene was 2 694 bp, encoding 897 amino acids. Functional domain analysis showed that the Lpin1 protein had highly conserved Lipin-N, Lipin-mid and LNS2 (Lipin/Ned1/Smp2) domains. Phylogenetic analysis revealed that the *M. amblycephala* Lpin1 was closely related to those of *Cyprinus carpio* and *Danio rerio*. The results of qPCR showed that *lpin1* was expressed at all stages during the embryonic development of *M. amblycephala*, and the expression level was the highest in the muscle of adult *M. amblycephala*. The results of WISH further revealed that *lpin1* was specifically expressed in the head and body segments during muscle functioning stage and heartbeating stage. Compared with the uninjured group, the *lpin1* expression was the highest at 48 h after mild injury and 216 h after severe injury, respectively, while the *pax7* expression was the highest at 144 h after mild injury and 96 h after severe injury, respectively. The above results indicated that *lpin1* gene may play an important role in muscle injury and repair of *M. amblycephala*.

Keywords *Megalobrama amblycephala*; *lpin1*; gene expression; muscle damage and repair

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