

研究论文

*BHC80*基因表达下调对斑马鱼胚胎心脏发育的损伤作用

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摘要: 有研究显示, 组蛋白去乙酰基酶复合物(BRAF-HDAC complex, BHC)组分BHC80基因敲除导致小鼠出生一天内死亡。为此, 本研究在斑马鱼中下调*BHC80*基因表达, 以深入研究其在生物体发育中的作用。根据斑马鱼*BHC80*序列, 设计合成吗啡啉修饰的反义寡核苷酸, 并将其显微注射到单细胞或双细胞期的野生型胚胎中, 并用RT-PCR方法验证其有效性。分析*BHC80*基因阻抑后对胚胎发育, 尤其心脏表型和功能的影响。结果显示, 吗啡啉修饰的反义寡核苷酸有效下调*BHC80*基因表达, 其对胚胎发育异常的影响呈剂量依赖性。*BHC80*基因表达下调的斑马鱼胚胎心脏出现多种异常的表型, 包括心房心室大小异常、环化不完全、严重者心脏发育呈管状、心搏减弱, 心率减慢、心室收缩分数降低。结果表明, *BHC80*基因表达下调致使胚胎心脏发育异常, 心功能受损, 这可能是*BHC80*基因敲除哺乳动物出生后很快死亡的重要原因之一, 为进一步阐明心脏发育机制提供了很好的研究工具。

关键词: 斑马鱼; 组蛋白去乙酰基酶复合物蛋白80; 吗啡啉反义寡核苷酸; 心脏发育

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Impaired effect of *BHC80* gene knock-down on the cardiac development in zebrafish

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Abstract: The effect of BHC80 (a component of BRAF-HDAC complex) on development was not well studied, because *BHC80* gene knock-out mice died in one day after birth. Interestingly, zebrafish embryos can live, even if their important organs like cardiac system has severe dysfunction, as 25%–40% O₂ are supplied through their skin. Therefore, a model of *BHC80* gene knock-down zebrafish embryos was established to explore the effect of BHC80 on the early embryonic development. *BHC80*-morpholino antisense oligonucleotides 2 (*BHC80*-MO2) was designed and injected into zebrafish embryos to interrupt the correct translation of *BHC80* mRNA at one or two cells stage, which was proved by RT-PCR analysis. Two control groups, including non-injection group and control-MO (con-MO) injection group, and four different doses of *BHC80*-MO2 injection groups, including 4 ng, 6 ng, 8 ng and 10 ng per embryo were set up. The embryonic heart phenotype and cardiac function were monitored, analyzed and compared between con-MO and *BHC80*-MO2 groups by fluorescence microscope in *vmhc:gfp* transgenic zebrafish which express green fluorescent protein in ventricle. The results showed that *BHC80*-MO2 microinjection effectively knocked down the *BHC80* gene expression, because the *BHC80*-MO2 group emerged a new 249 bp band which reduced 51 bp compared to 300 bp band of con-MO group in RT-PCR analysis, and the 51 bp was the exon 10. The abnormal embryo rate rose with the increase of *BHC80*-MO2 dosage. The proper *BHC80*-MO2 injection dosage was 8 ng per embryo, as minor embryos had abnormal phenotype in 4 ng and 6 ng per embryo groups and most embryos died in 10

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ng per embryo group. *BHC80*-MO2 embryos exhibited abnormal cardiac phenotype, including imbalance of the proportion of heart ventricle to atrium, incomplete D-loop, even tubular heart, slow heart rates and cardiac dysfunction. The results from a model of *BHC80* gene knock-down zebrafish embryos show that the abnormal cardiac phenotype and cardiac dysfunction of *BHC80*-MO2 embryos may be one of the probable reasons for the *BHC80* gene knock-out mice death, which would provide a good research model to clarify the mechanism of cardiac development.

Key words: zebrafish; *BHC80*; morpholino antisense oligonucleotides; cardiac development

BHC80 是组蛋白去乙酰基酶复合物 (BRF-HDAC complex, BHC) 的骨架蛋白, 凭借植物同源结构域 (plant homeodomain, PHD) 能够结合 BHC 的其它组分^[1]。BHC 可表达于非神经细胞, 参与抑制神经特异性基因的转录, 参与靶基因启动子转录活化和抑制状态之间的转变, 该过程包括组蛋白 H3 的第 9 位赖氨酸 (H3K9) 去乙酰化、甲基化和组蛋白 H3 的第 4 位赖氨酸 (H3K4) 去甲基化^[2,3]。*BHC80* 基因敲除导致小鼠出生一天内死亡^[4], 难以研究其功能, 而斑马鱼氧气供应的 25%~40% 由体表渗透, 其存活可不依赖造血和心血管系统, 并且斑马鱼仔鱼通体透明, 可用肉眼直接观测表型变化。斑马鱼基因组, 尤其是编码基因与哺乳动物同源性高达 75%, 因此斑马鱼是研究心血管早期发育的良好模式生物^[5]。吗啡啉修饰的反义寡核苷酸技术是在斑马鱼中应用最广的基因下调技术, 其原理是抑制 mRNA 的翻译或剪接^[6]。本研究以斑马鱼为研究对象, 用吗啡啉修饰的反义寡核苷酸技术, 通过受精卵显微注射, 观察抑制 *BHC80* 基因表达对心脏发育的影响, 以利于进一步阐明人类心脏发育的基因调控机理。

1 材料与方法

1.1 实验动物 野生型斑马鱼和绿色荧光蛋白标记的心室肌球蛋白重链 (*vmhc:gfp*) 转基因斑马鱼胚胎来自复旦大学分子医学教育部重点实验室斑马鱼 (AB* 品系) 养殖基地, 依照 *Zebrafish Book* 方法进行喂养及产卵操作^[7]。用孵化液 (60 mg/mL 海盐) 28 °C 孵化受精卵, 用 0.003% 苯硫脲处理受精后 24 h (24 h post fertilization, 24 hpf) 以上的胚胎, 以防止黑色素形成。

1.2 吗啡啉反义寡核苷酸技术 有两种吗啡啉反义寡核苷酸 (morpholino antisense oligonucleotides, MO): 起始密码 MO (MO1) 和剪接 MO (MO2)。MO1 结合靶基因起始密码区域, 阻止靶基因 mRNA 翻译; MO2 结合靶基因核内不均一 RNA (heteroge-

neous nuclear RNA, hnRNA), 抑制其正确剪接, 从而使 *BHC80* 基因表达出无义蛋白质或错义蛋白质。我们针对斑马鱼 *BHC80* 基因设计了 *BHC80*-MO2 序列, 该序列结合第九内含子和第十外显子交界部位, 使剪接后的 mRNA 缺少第十外显子。*BHC80*-MO2 序列: 5'-AGTTTCTGATGGAAACAGCAAAGGA-3'。对照反义寡核苷酸序列 (control-MO, con-MO): 5'-CCT-CTTACCTCAGTTACAATTATA-3'。

1.3 显微注射 在单细胞或双细胞期的野生型胚胎中注射 MO, 用 RT-PCR 方法验证 MO 抑制 *BHC80* 基因的有效性。PCR 反应条件为: 94 °C 5 min; 94 °C 45 s, 55 °C 45 s, 72 °C 45 s, 30 个循环; 72 °C 8 min。取 10 μL 反应液, 2% 琼脂糖凝胶电泳鉴定产物。正向引物: 5'-TGTGAAACCACAGG-GAGGAC-3', 反向引物: 5'-CTCTTCCTGGTGC-CTTGATG-3'。为了检测 *BHC* 基因本身在斑马鱼心脏的表达谱, 用上述 RT-PCR 方法对 24、48 和 72 hpf 的斑马鱼胚胎心脏中 *BHC80* 基因表达情况进行检测。

1.4 实验分组 设置 4 个不同剂量的 *BHC80*-MO2 注射组, 分别为 4 ng/ 胚胎、6 ng/ 胚胎、8 ng/ 胚胎和 10 ng/ 胚胎。设置两个对照组: 未注射组和 con-MO 注射组。

1.5 形态学观察 在斑马鱼胚胎发育过程各观察时点计数胚胎存活情况及畸胎数量, 并用 Olympus 解剖显微镜拍照及录像, 观察胚胎发育及心脏形态功能情况。胚胎发育异常的判定标准为: 头部畸小、心前区水肿、心搏减弱和心率减慢。

1.6 心室收缩分数的测定 用摄像机 (JVC, TK-C1381) 拍摄胚胎心脏, 按以下公式计算心室收缩分数 (ventricular shortening fraction, VSF)^[8]。VSF = (舒张期心室长径 - 收缩期心室长径) / 舒张期心室长径 × 100%

1.7 数据分析 实验重复 3 次, 数据为 mean ± SD。应用 SPSS19 软件统计数据, 用双侧 *t* 检验比

较两组间均值,用卡方检验比较两组间率, $P < 0.05$ 时认为差异有统计学意义。

2 结果

2.1 *BHC80* 基因在斑马鱼心脏表达谱

为了研究 *BHC80* 基因本身在斑马鱼心脏发育过程中的表达情况,我们采用 RT-PCR 的方法对 24、48、72 hpf 的斑马鱼胚胎心脏进行分析,结果显示 *BHC80* 基因在斑马鱼发育早期从 24 hpf 到 72 hpf 持续表达(图 1),说明 *BHC80* 是母系来源的基因,在胚胎心脏发育中起重要作用。

2.2 *BHC80*-MO2 的效率验证

注射吗啡啉修饰的反义寡核苷酸是一种已经建立的良好干扰目标 mRNA 翻译的方法^[9]。*BHC80*-MO2 的效果用 RT-PCR 方法验证,结果显示 *BHC80*-MO2 能够有效抑制内源性 *BHC80* 基因表达。PCR 正向引物在第九外显子区域,反向引物在第十二外显子区域,正常内源性 *BHC80* 基因的 PCR 产物为 300 bp, *BHC80* 基因被 *BHC80*-MO2 剪切掉第十外显子(51 bp)后的 PCR 产物为 249 bp (图 2)。

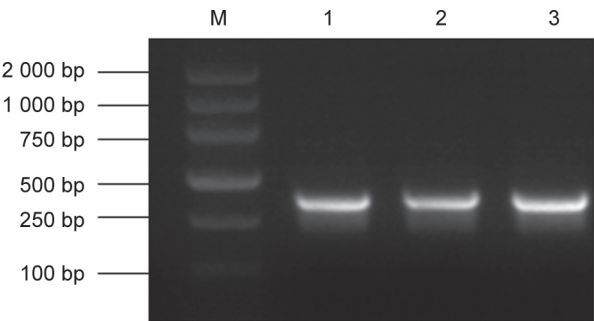


图 1. *BHC80*基因在心脏的表达
Fig. 1. The expression of *BHC80* gene in the heart of zebrafish embryos. M: Marker; 1–3: RT-PCR analysis of *BHC80* gene expression in the heart of zebrafish embryos at 24, 48 and 72 h post fertilization (hpf).

2.3 不同剂量 *BHC80*-MO2 对斑马鱼胚胎发育的影响

我们统计了 48 hpf 时各组胚胎存活和致畸情况(表 1),显示随着 *BHC80*-MO2 注射剂量的增加,胚胎的死亡率和异常率呈上升趋势。相对于 8 ng con-MO 组,8 ng *BHC80*-MO2 组出现胚胎发育异常,即头部畸形、心前区水肿、心搏减弱和心率减慢(图 3)。8 ng *BHC80*-MO2 组中 95 个胚胎出现异常,4 个胚胎正常;8 ng con-MO 组中 2 个胚胎出现异常,115 个胚胎正常,两组异常率用卡方检验比较有明显差异($P < 0.05$)(表 1)。10 ng *BHC80*-MO2 组胚胎到 48 hpf 时死亡率高达 75%,且存活者畸形严重;未注射组和 con-MO 注射组胚胎未见明显畸形(表 1)。8 ng *BHC80*-MO2 组相对于 4 ng *BHC80*-MO2 组和 6 ng *BHC80*-MO2 组有更高的异常率,而相对于 10 ng *BHC80*-MO2 组有更少的死亡率(表 1),故 8 ng *BHC80*-MO2 用来研究心脏表型和功能最为合适,本研究后续心脏表型和功能实验都采用 8 ng *BHC80*-MO2。

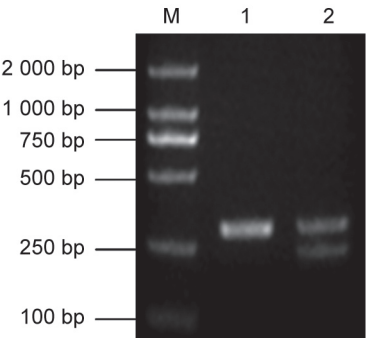


图 2. RT-PCR方法分析受精后48 h (48 h post fertilization, 48 hpf)胚胎*BHC80*基因的表达
Fig. 2. RT-PCR analysis of *BHC80* gene expression of embryos at 48 h post fertilization (48 hpf). M: Marker; 1: Injection of standard control-MO at 8 ng per embryo produced 300 bp band; 2: Injection of *BHC80*-MO2 at 8 ng per embryo produced 300 and 249 bp bands.

表1. 不同剂量*BHC80*-MO2对斑马鱼胚胎发育的影响
Table 1. Effect of *BHC80*-MO2 on zebrafish embryonic development

	Wild type	Con-MO 8 ng	<i>BHC80</i> -MO2			
			4 ng	6 ng	8 ng	10 ng
Normal	118 (98.3%)	115 (95.8%)	103 (85.8%)	32 (26.7%)	4 (3.3%)	5 (4.2%)
Abnormal	0	2 (1.7%)	11 (9.2%)	74 (61.6%)	95 (79.2%)	25 (20.8%)
Death	2 (1.7%)	3 (2.5%)	6 (5%)	14 (11.7%)	21 (17.5%)	90 (75%)
Total	120	120	120	120	120	120

2.4 *BHC80* 基因表达下调对斑马鱼心脏表型的影响

在正常野生型 *vmhc:gfp* 转基因斑马鱼 72 hpf 时, 心脏早已完成向右环化, 心室位于心房的右侧, 心血管循环系统功能完善。与 con-MO 注射对照组相比, 8 ng *BHC80*-MO2 注射 *vmhc:gfp* 转基因斑马鱼后的 72 hpf 胚胎心脏, 表现出心房心室大小异常 (图

4A, 4B), 心前区水肿、心脏发育呈管状、环化不完全 (图 4C)。

2.5 *BHC80* 基因表达下调导致斑马鱼胚胎心脏功能受损

Con-MO 注射对照组胚胎 24 hpf 时心脏搏动有力, 心率为 (93 ± 5) 次/min ($n = 20$); *BHC80* 基因

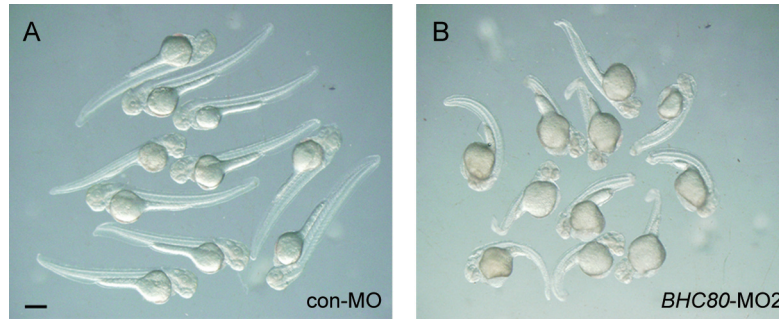


图 3. *BHC80* 基因表达下调的胚胎表型

Fig. 3. The phenotype of *BHC80* gene knock-down embryos. A: Injection of standard control-MO at 8 ng per embryo. B: Injection of *BHC80*-MO2 at 8 ng per embryo. Scale bar, 250 μ m.

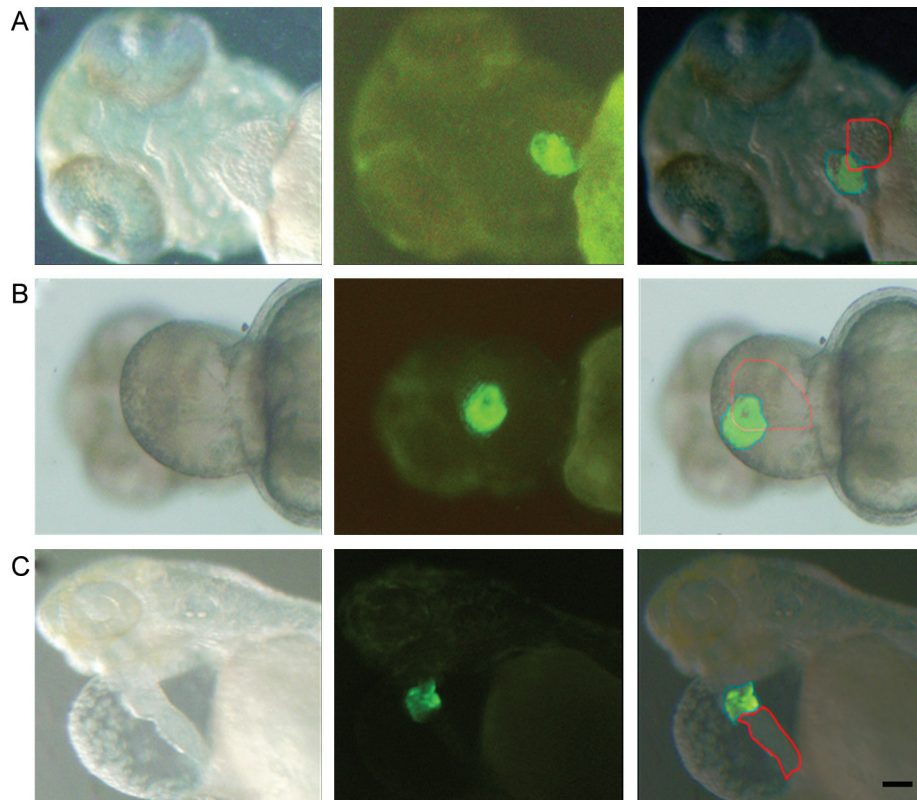


图 4. *BHC80* 基因表达下调的 *vmhc:gfp* 转基因斑马鱼 72 hpf 胚胎心脏表型

Fig. 4. The heart phenotype of *vmhc:gfp* transgenic zebrafish embryos at 72 hpf with *BHC80* gene knock-down. A: *vmhc:gfp* embryos injected with 8 ng standard control-MO. B, C: *vmhc:gfp* embryos injected with 8 ng *BHC80*-MO2. The first column was shown under white light, the second was shown under fluorescence microscope, and the third was merged by the first and second columns. The atrium was outlined in red, and the ventricle expressed green fluorescent protein and was outlined in blue. Scale bar, 50 μ m.

抑制组 24 hpf 胚胎心脏搏动无力, 心率为 (64 ± 8) 次/min ($n = 20$), 与对照组相比有明显差异 ($P < 0.05$)。心脏发育到 48 hpf 后心率差异更加明显, con-MO 注射对照组心率为 (153 ± 6) 次/min ($n = 20$), *BHC80* 基因抑制组的心率为 (71 ± 10) 次/min ($n = 20$), 两组相比有明显差异 ($P < 0.05$) (图 5)。

心室收缩分数是衡量心脏收缩强度最敏感的指标之一。在 48 hpf 时, 对照组胚胎心室收缩分数为 $(33.9 \pm 4.6)\%$ ($n = 10$), 而 *BHC80* 基因抑制组胚胎心室收缩分数为 $(18.4 \pm 5.3)\%$, 与对照组相比有明显差异 ($P < 0.05$) (图 6)。

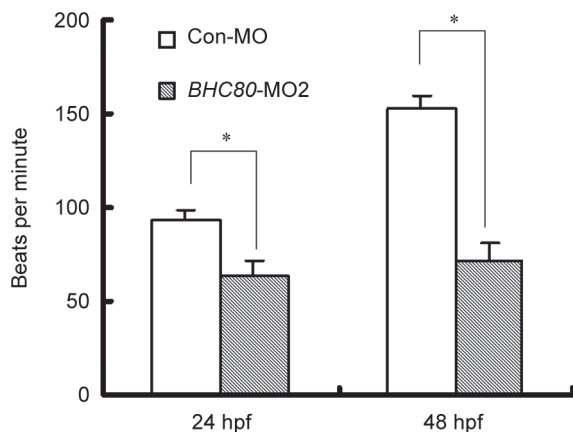


图 5. *BHC80*基因表达下调组与对照组心率比较

Fig. 5. Difference of heart rate between *BHC80* gene knock-down and control embryos. 24 hpf: 24 h post fertilization; 48 hpf: 48 h post fertilization. Mean $\pm$ SD, $n = 20$. * $P < 0.05$.

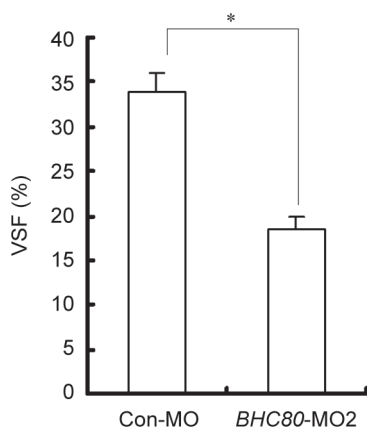


图 6. 48 hpf 胚胎 *BHC80* 基因表达下调组与对照组心室收缩分数比较

Fig. 6. Comparison of ventricular shortening fraction (VSF) in *BHC80* knock-down and control embryos at 48 hpf. Mean $\pm$ SD, $n = 20$. * $P < 0.05$.

3 讨论

Iwase 等^[4]研究指出 *BHC80* 基因缺陷小鼠在出生一天内死亡是因为不能有效吸吮母乳, 而其神经和非神经组织未表现出异常。然而, 心脏作为维持生命的重要器官之一, Iwase 等只是指出 *BHC80* 基因在心脏中表达, 但并未深入研究 *BHC80* 基因缺陷小鼠的心脏是否发育异常。因而, *BHC80* 基因缺陷与心脏发育之间的关系值得进一步研究, 而心脏发育异常在小鼠等模式生物中易于致死, 但斑马鱼氧气供应的 25%~40% 由体表渗透, 其存活可不依赖心血管系统, 所以, 我们选择斑马鱼作为 *BHC80* 基因缺陷的研究模型。

为了建立 *BHC80* 基因缺陷斑马鱼, 我们设计了针对斑马鱼 *BHC80* 基因第十外显子的吗啡啉反义寡核苷酸 *BHC80*-MO2, 第十外显子是 *BHC80* PHD 结构域的重要组成部分, *BHC80* PHD 结构域结合未甲基化的 H3K4 (H3K4me0), 以保持靶基因的转录抑制^[10]。第十外显子被 *BHC80*-MO2 剪切掉后, PHD 结构域正常功能丧失, 其靶基因的转录不再受到抑制, 这可能是导致胚胎发育异常的重要原因。

本研究结果显示, *BHC80* 基因缺陷斑马鱼胚胎发育异常包括头部畸形、心前区水肿、心脏发育异常、体节弯曲, 其中心脏环化不完全甚至无明显向右环化。斑马鱼心脏向右环化, 使心室在心房的右前侧, 心房心室在侧面观上大部分重叠, 这对于心脏各腔室及大血管的正确定向和排列有重要意义, 内脏异位综合征及某些单纯心脏畸形均可能与环化异常有关^[11, 12]。人类先天性心脏病, 是小儿最常见的先天畸形之一, 活产婴儿发病率为 0.6%~1%, 在死胎和自然流产胎儿中则分别占到 10% 和 20%^[13, 14], 心脏发育机制复杂, 目前仍不十分清楚。本实验中, *BHC80* 在斑马鱼发育早期从 24 hpf 到 72 hpf 持续表达, 说明 *BHC80* 基因是母系来源的基因, 在胚胎心脏发育中起重要作用; *BHC80* 基因缺乏的胚胎出现心脏环化不完全以及心率减慢、心室收缩分数降低等心功能不全的表现, 这可能是 *BHC80* 基因缺陷小鼠出生一天内死亡的重要原因之一, 对进一步阐明心脏发育机制十分重要。

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