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论文题目：关键应激蛋白 HSP90 调控涡虫再生能力的新发现

Title: A **new discovery of the key stress protein HSP90 in regulating the regenerative ability of the planarian**

【全英文论文附后】

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作者：邹普越 曹楚林 胡瀚丹

【摘要】涡虫具备超强的再生能力，但其再生机制还不清楚。HSP90 是一种高度保守的应激蛋白，为了探索 HSP90 对涡虫再生的调控作用，首先切割涡虫后用荧光定量 PCR 技术分析，发现 HSP90 在涡虫切后再生的早期应激反应中表达量明显上升。进一步喂食 dsRNA 干扰 HSP90 表达，测定涡虫再生速率，与对照组相比，发现干扰组涡虫再生能力明显降低，切后 6h 仍创伤面不能应激收缩，再生头部眼点出现时间延迟了 24h，干扰组涡虫再生 6 天也没能形成完整个体。测量涡虫再生过程中长/宽比值变化，发现干扰组涡虫再生速率极其缓慢。

荧光定量 PCR 分析涡虫干细胞的 PCNA 基因表达水平，表明干扰组涡虫再生能力降低与其体内干细胞增殖减少密切相关。最后，通过免疫组化技术，发现干扰组涡虫中再生的脑神经节前端和腹神经索末端再生 6 天后仍未能完全闭合，而对照组涡虫神经系统修复正常。本实验结果说明 HSP90 既能调控涡虫个体再生，也能调控涡虫神经系统创伤修复，是调控涡虫再生的一个关键蛋白。本研究结果有助于理解和探索再生以及神经损伤修复这一世界性难题。

关键词：HSP90，涡虫，再生，神经修复，RNA 干扰

A new discovery of the key stress protein HSP90 in regulating the regenerative ability of the planarian

Abstract: Planarian is a kind of unique animal, which exhibit an extraordinary ability to regenerate lost body parts. However, its mechanism of regeneration is unclear.
HSP90 is a highly conserved stress protein. In order to explore the relationship between HSP90 and the regeneration of the planarian, several experiments were carried out in this study.

After amputation, qPCR results showed that the expression of HSP90 was obviously increased in the early planarian stress response. RNA interference by feeding in vitro-synthesized HSP90 dsRNA to planarians, and then planarians were amputated into 2 fragments in pre-pharynx at the 24th hour after the last feeding. It was found that the regenerative ability of the planarians detected in the interference group was significantly lower than that of the control group. After amputation, the wound surface could not contract at the 6th hour, the appearance of eyespots on the regenerated head was delayed 24hrs, and the planarian of the interference group did not regenerate into a complete worm after 6 days, on the contrary, the planarian of the control group regenerated into a complete individual. The regeneration rate was measured through the ratio of length and width. It was found that during the process of regeneration, the ratio of the length/width observed in the inference group was significantly lower than in the control group.

The expression of *PCNA* gene by qPCR showed that the regenerative ability of the planarian was closely related to the decrease of the stem cell proliferation in the interference group. Through immunofluorescence technique, it was found that the end of the brain ganglion and the ventral nerve cord of the interference planarian group were not completely closed on the 6th day of regeneration, while that of the control group were regenerated normally.

These results indicated that HSP90 can not only regulate the individual regeneration of the planarian, but also the healing of its nervous system, so it is the key protein in regulating the regeneration of the planarian. This study helps us to understand and explore the mechanism of the planarian regeneration and the repair of the nervous system, which has been a worldwide problem.

Key words: HSP90; planarian; regeneration; RNA-interference; nervous repair

目 录

1. 项目由来与背景

在参加我们学校邀请校外专家所做的一场学术报告中，专家讲到了一种名叫涡虫的动物，它能砍头长头，切尾长尾，更惊奇的是，1898 年，遗传学家摩尔根切下涡虫整体重量 1/279 的小块组织都能再生为一个完整的个体，被誉为“切不死的动物”(Morgan, 1898)。涡虫这种超强的再生能力立刻引起了我们强烈的好奇：小小的涡虫为什么有如此强大的再生能力？其再生的秘密在哪里？人和其它很多动物为什么不能像涡虫那样再生？带着这些问题和好奇，我们在百度搜索了涡虫相关信息，对这种神奇的动物有了初步了解。

在确定要做涡虫再生实验并请教指导老师和川大专家后，我们知道了要从专业数据库查找涡虫再生相关的中文，甚至外文文献，才更可靠。通过阅读文献，我们了解到不同涡虫生活环境不一样。淡水涡虫隶属于扁形动物门(Platyhelminthes)、涡虫纲(Turbellaria) (陈广文 等，2000)，身体长 10mm 左右。在我国分布很广的淡水涡虫是日本三角涡虫(*Dugesia japonica*)，身体扁长，两侧对称，有头、尾、躯干、背腹之分，头呈三角形，背面两侧略突的耳突内侧有一对黑色眼点，腹部中间有口和咽部，体后端稍尖(图 1)。

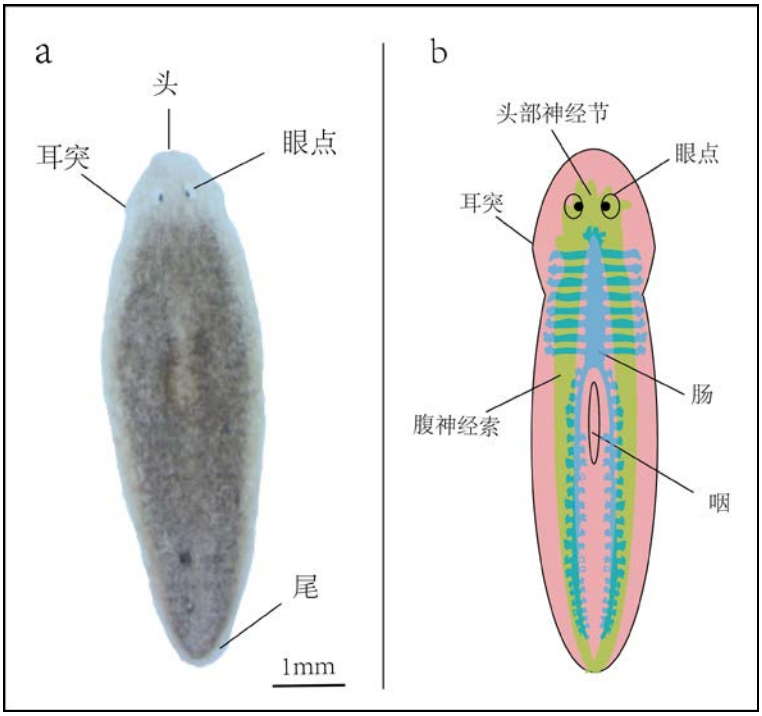

图 1 涡虫形态和结构图

a.体视显微镜下的涡虫 b.涡虫结构简图

从遗传学家摩尔根研究涡虫再生开始，100 多年来，科学家们对涡虫的再生能力进行了很多研究，但是由于缺乏更好的研究技术，比如说分子生物学技术，早期研究主要是对涡虫再生现象的描述。而真正探索涡虫再生秘密的研究是在近些年来，利用 RNA 干扰 (RNA interference, RNAi) 等技术，才发现了一些可能与涡虫再生相关的基因和信号通路(Newmark et al., 2002)。

涡虫惊人的再生能力与其体内有丰富的成体干细胞密切相关，这类细胞通过迁移、增殖和分化，使受损的涡虫可以形成一个完整的个体。涡虫一旦受到创伤就会引发应激反应，随后通过各种信号调动体内干细胞的迁移、增殖与分化等生理过程。那么，创伤是否会激发涡虫体内干细胞某些基因的表达来刺激它的再生呢？好奇之心又一次激发了我们探索的热情，我们查找了与创伤、刺激和生长相关的基因，发现了一个重要基因 Hsp90 (heat shock protein, Hsp90)。该基因编码的蛋白广泛存在于原核和真核生物细胞中，结构高度保守，因为分子量约 90 kDa，并在机体或细胞所处环境温度升高时而表达，所以得名热激蛋白 HSP90。后来人们还发现，除了温度刺激外，当受到其它物理、化学或生物因子刺激时，HSP90 也会高表达，并通过传导信号，使机体做出正确应答，所以又称为应激蛋白(李艳光 等，2013)。此外，HSP90 还因为参与细胞的生长、发育和分化，与肿瘤、真菌耐药性等有较为密切的关系而备受关注(张丽萍 等，2014；王琪林 等，2011)。进一步查阅文献，我们发现有关涡虫 HSP90 的研究很少。有报道克隆该基因，并分析在热和高浓度重金属镉环境中，其表达情况，并发现 HSP90 有保护涡虫胃皮细胞的作用(马克学 等 2013；程方方 等，2016)。我们整个实验于 2017 年 8 月完成，2018 年 1 月查新报告完成，没有查到有关 HSP90 对涡虫再生的调控作用研究。

提出科学假设与问题：

我们推测，有没有可能在涡虫被切割之后，组织缺失作为一种刺激引起了涡虫应激反应，随后导致涡虫 HSP90 高表达，HSP90 的表达就是涡虫再生的关键前提和重要信号呢？如果是这样，可通过什么实验手段加以证明呢？为了一探究竟，在指导老师的帮助下，通过查阅文献和请教分子生物学专家，觉得可以用 RNA 干扰方法去涡虫体内 HSP90 的表达，再观察涡虫再生过程是否会受到抑制。就这样，我们查阅了 RNA 干扰技术资料并设计了实验方案，开始了实验。

2.实验材料、设计与方法

2. 1 实验材料与主要设备

日本三角涡虫(*Dugesia japonica*)：清华大学 XX 教授提供，以下简称涡虫。

DH5α 感受态细菌购于擎科生物公司；pMD 19-T 载体购于 TaKaRa 公司。

成都七中提供：带数码相机的体式显微镜(SMZ-168，Motic)、PCR 仪(C1000Touch，Bio-Rad)，实时荧光定量 PCR 仪(GFX Connect，Bio-Rad)，冷冻离心机(Micro 17R，Thermo-Fisher)、电泳槽(Mini-sub cell GT，Bio-Rad)、高灵敏度化学发光成像系统(ChemiDoc XRS^{+}，Bio-Rad)等。其它试剂与药品购于 TaKaRa 等公司。

四川大学提供：倒置荧光显微镜(IX73, Olympus)

2. 2 实验设计与流程

有文献报道，要干扰涡虫基因表达，可以喂食涡虫双链 RNA (double-stranded RNA, dsRNA)来达到目的(Rouhana et al., 2013；吴雪 等，2013)，而要看基因是否被干扰，可用荧光定量 PCR 方法(qPCR)来检测。于是，我们通过分子生物学手段合成 HSP90 的 dsRNA，先干扰 HSP90 的表达，然后检测涡虫再生及神经系统修复情况。实验设计及主要流程示意如图 2。

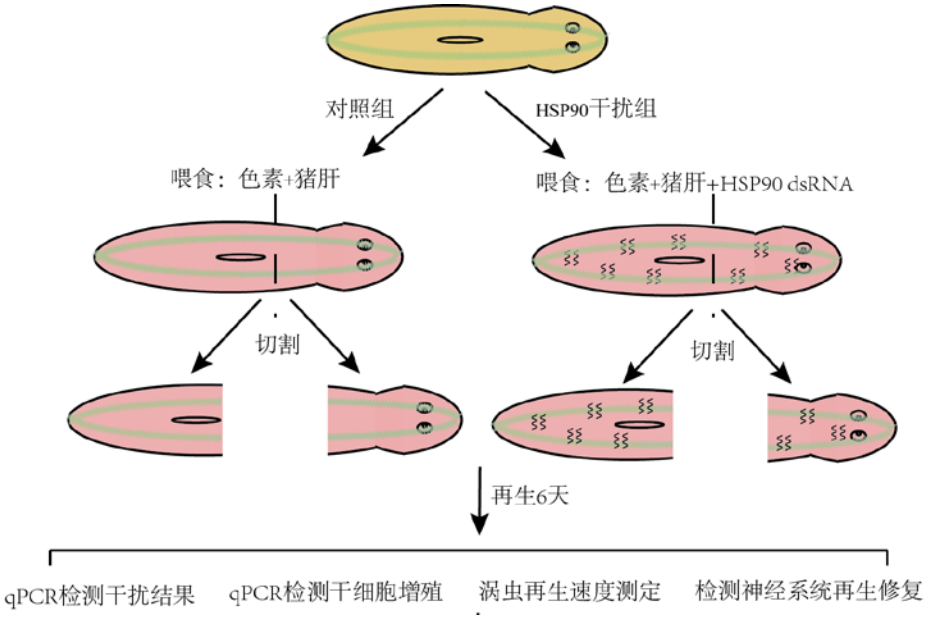

图 2. 实验主要流程示意图（喂食色素后涡虫变色）

2.3 实验方法

2. 3. 1 涡虫喂养与切割

日本三角涡虫在室温下喂养于盛饮用水的玻璃碗中，每 1~2 天喂食猪肝一次，喂后换水。实验前挑取长度约 1cm 的涡虫提前饥饿 5~7 天进行 RNA 干扰、切割等实验。为避免涡虫蠕动，切割时将涡虫滴在载玻片上，载玻片放在碎冰上。

2. 3. 2 喂食 dsRNA

对照组与实验组涡虫在实验前饥饿一周左右。每一处理组涡虫数量为 5 只。在培养皿中，用小刀将猪肝刮成泥浆，然后用移液器吸 200μl 猪肝浆于 EP 管中，按 10μl 猪肝浆：1μg dsRNA 的比例加入 dsRNA。再向 EP 管中按照总体积 1%的比例加入食用色素(红色)，混匀，离心 30s，以形成更密集的团，然后加到有涡虫的培养皿中，让涡虫在黑暗环境下进食 1h。这样每隔一天喂一次，共喂 3 次(Rouhana et al., 2013)。喂食后将涡虫放在体式显微镜下观察消化道是否变红以指示 dsRNA 摄入与否(图 3)。

考虑到 HSP90 对外界理化及生物因子的刺激比较敏感，为了让对照组涡虫 HSP90 的表达更接近自然状态，参照国际权威文章，对照组用等体积水代替随机 dsRNA 喂食 (Newmark et al., 2003；Almuedo-Castillo M et al., 2014)。事实上，我们用增强型绿色荧光蛋白(eGFP) dsRNA 喂食涡虫后，发现与喂水相比，会引起 HSP90 高表达(见实验记录)。因此，本探究没有用喂食随机 dsRNA 作为对照。

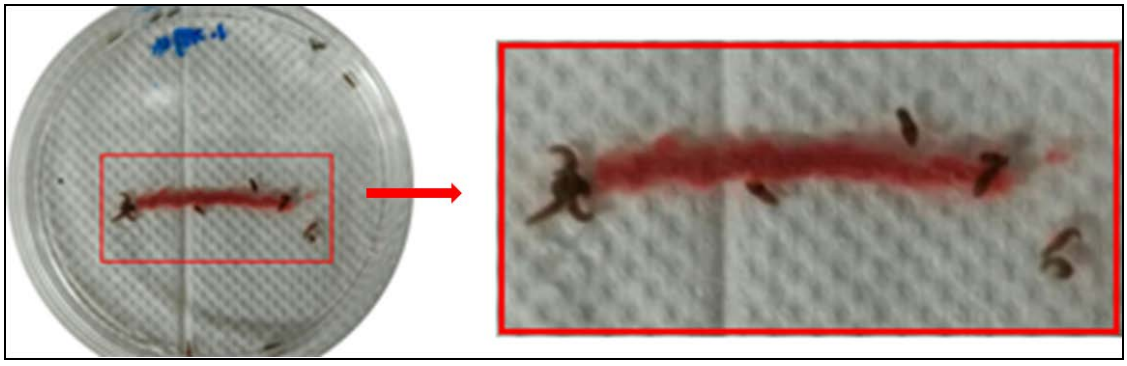

图 3. dsRNA 干扰喂食法(右图为左图的放大，红色为含色素和 dsRNA 的猪肝泥)

2. 3. 3 RNA 的提取与反转录

取 5 只涡虫放在灭过菌的 1.5mL EP 管，加入 1mL RNA 提取试剂 RNAiso

plus，用枪头反复吸打涡虫至小碎屑。参照《分子生物学实验》(吴建祥 等，2014)提取涡虫总 RNA，保存于-80℃超低温冰箱中备用。

反转录时，根据反转录试剂盒说明书(TaKaRa 公司)，用提取的 1μg 涡虫 RNA 在 37℃ 15min、85℃ 5s 条件下反转录生成 cDNA，-20℃保存备用。

2. 3. 4 DNA 琼脂糖凝胶电泳及胶回收

参照《分子生物学实验》(吴建祥 等，2014)进行 DNA 琼脂糖凝胶电泳。DNA 胶回收方法参照天根公司试剂盒说明书进行。

2. 3. 5 引物设计

从美国国立生物技术信息中心(NCBI)数据库中下载涡虫相关基因序列，然后利用 Primer Premier 5 引物软件设计基因扩增引物。

2. 3. 6 PCR 及 qPCR 方法

利用基因引物，以涡虫反转录 cDNA 为模板，按照 TaKaRa rTaq 聚合酶说明书，常规 PCR 方法扩增复制基因。

利用设计的 qPCR 引物和涡虫反转录 cDNA，根据 Inovogene 公司 qPCR 试剂盒说明书进行基因表达扩增，并通过 ΔΔCt 法定量分析。

2. 3. 7 HSP90 双链 RNA 的合成

为了方便体外 T7 RNA 聚合酶转录出 HSP90 双链 RNA，用带 T7 启动子的引物从 cDNA 上 PCR 扩增 HSP90 基因。由于 dsRNA 需要量大，为了避免浪费 cDNA，将得到的 HSP90 连到 pMD 19-T 载体上(参照 TaKaRa 公司提供的方法)，然后转化大肠杆菌 DH5α，涂布于含 100μg /mL 氨苄青霉素的 LBA 平板上，37℃培养过夜后挑取单克隆菌落做 PCR 鉴定，送华大基因公司测序。测序确证后从重组质粒上 PCR 扩增、胶回收，然后体外转录生成 dsRNA (按 Thermo Fisher 体外 RNA 转录试剂盒说明书进行)。将得到的 dsRNA 与猪肝浆混合，喂食涡虫(图 4)。质粒提取方法参照《分子生物学实验》(吴建祥 等，2014)。

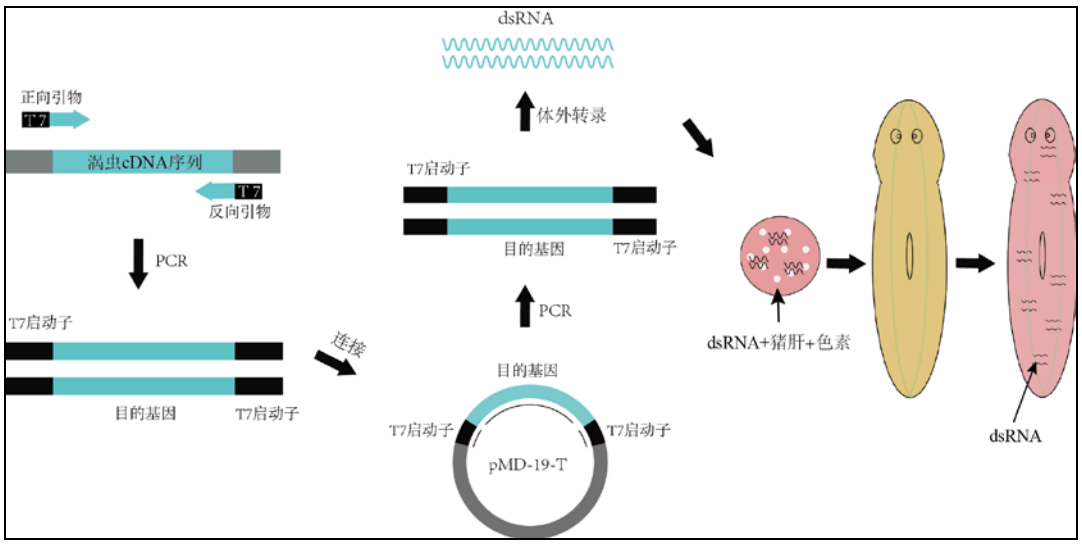

图 4. dsRNA 体外转录及体内干扰的示意图

2. 3. 8 涡虫再生速度测定

dsRNA 喂食 3 次进行干扰后第 3 天，在咽前将涡虫切成两半，然后将头部和尾部放在 6 孔板中，在 0h、6h、1~6 天时体视显微镜观察拍照记录再生情况。

涡虫再生速度测定：在涡虫自然伸展状态下(放置冰上)，体视显微镜下拍照记录，然后根据标尺长度用 photoshop 软件换算涡虫长度与宽度（宽度测量位置：咽前），计算长/宽比值。用长/宽比值描述涡虫的再生速度(Tu et al., 2012)。

2. 3. 9 涡虫神经系统修复的免疫组化分析

涡虫切后 0h 以及再生 2 天、6 天时，对照组和 HSP90 干扰组用神经系统特异性标记蛋白的抗体 anti-SYNORF1(3C11) (购于 DSHB 公司)进行免疫组化分析，包括对涡虫进行固定、脱水、通透、水化、封闭及一抗、二抗标记等，最后在荧光显微镜下观察。具体步骤参照文献(陈旭辉，2014)。

3. 实验结果与分析

3.1 见识涡虫强大的再生能力

初次见到涡虫这个弱小而强大生命的时候，好奇心驱使我们先亲身体验一番传说中涡虫强大的再生能力，于是用不同方式切涡虫，看创伤是否能够得到再生和修复。先是将涡虫头部一端切开不同程度的伤口，尾部保留完整，在体式显微镜下观察并拍照记录再生过程；其次是尾部一端切开不同程度伤口，头部保留完

整；最后是横向切为三段，前端斜切，后端平切，观察中间段是否真的会按照原来的方向长出相应的头部和尾部。再生观察结果见图 5。

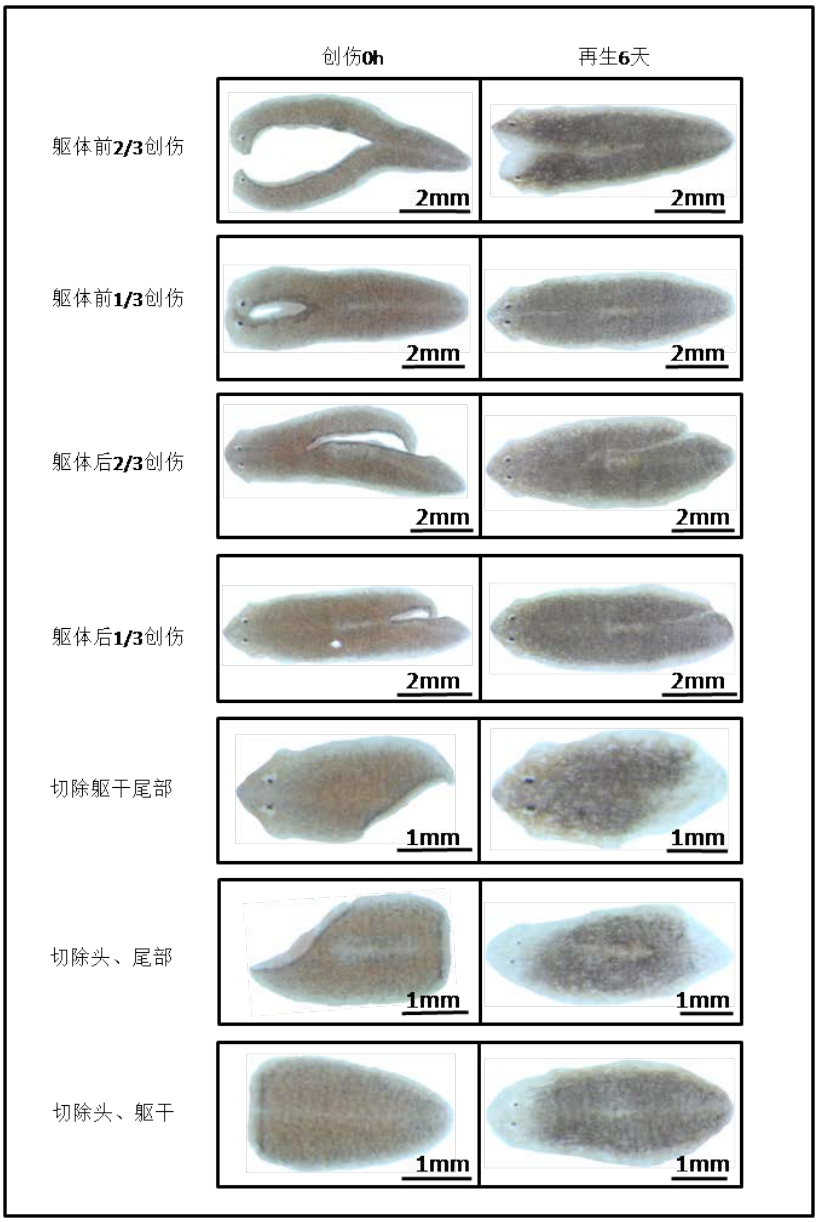

图 5.不同方式切后的涡虫再生探索图

当从头部创伤达身体长度 2/3 时，再生 6 天后竟然长出了两个头，而从头部创伤达身体长度 1/3 时，再生 6 天后伤口却完整的愈合了；尾部再生中，尾部伤口长度不管是躯体长度的 2/3 还是 1/3，再生 6 天后都不能长出两个尾。

为了观察涡虫被切成 3 段后，中间部分的再生是否有严格的极性，即躯干前端再生出来的是头部，躯干后端再生出来的是尾部，于是将前端斜切、尾端水平切，以便区分。再生 6 天后，发现涡虫再生有严格的极性，躯干前端再生出的依

然是头，后端再生出的依然是尾。这一体验让我们好奇如此小的动物，再生是如何发生、如何得到精确调控的。

3. 2 HSP90 是调控涡虫个体再生能力大小的关键基因

3. 2. 1 涡虫被切断后 HSP90 表达量明显增加

为了看 HSP90 表达变化，用 qPCR 技术来定量分析。指导老师教会我们如何从美国国立生物技术信息中心(NCBI)数据库中下载基因序列，以及引物设计软件的使用。HSP90 正向引物序列为 5’-TTGTCCTAAACGTGCTCCA-’3，反向引物序列为 5’-TCACGAAATTCAAATACTCTGG-’3。从图 6a 可见，涡虫切后再生 6h、12h，HSP90 的表达量明显增加，达到对照组的 2 倍左右，差异很显著；在切后 24h，表达量恢复如初。可见涡虫切后的应激反应中，HSP90 可能起到了很重要的作用。HSP90 基因 qPCR 扩增曲线正常(图 6b)、溶解曲线峰值单一(图 6c)，说明基因扩增特异性很好。有研究显示，在切割后，涡虫肌肉会快速收缩，以减小创伤面积，成体干细胞也会迅速增殖，HSP90 在切后 6h，表达量明显增加(马克学 等，2013)。由此更加坚定了我们的推测，即 HSP90 做为创伤后应激反应的关键蛋白，它的表达量升高可能对涡虫再生过程的启动具有至关重要的作用，于是我们决定进一步研究 HSP90 在再生过程中的作用。

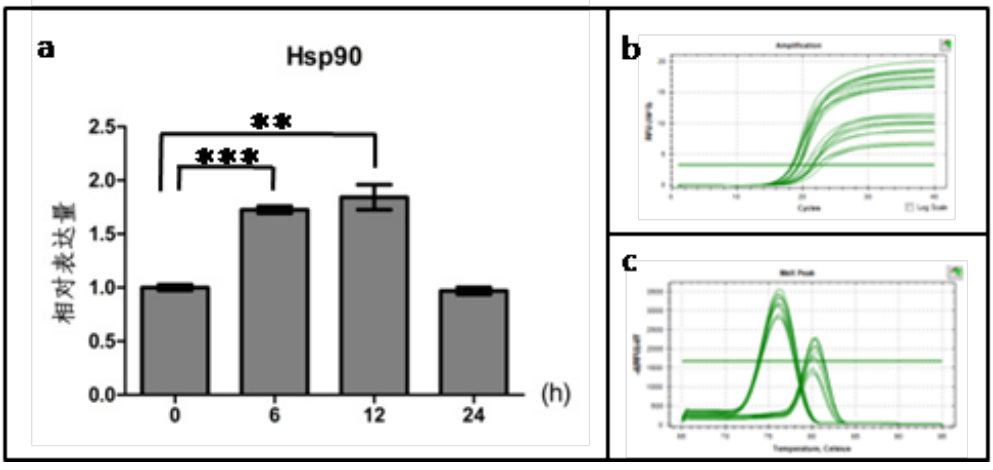

图 6. 涡虫切后不同时间 HSP90 荧光定量 PCR 结果

a 为创伤后 HSP90 基因不同时间表达变化图；b 表示 qPCR 的扩增曲线；c 为 qPCR 溶解曲线；实验重复 3 次；***表示 $P<0.001$，**表示 $P<0.01$

3. 2. 2 利用 dsRNA 干扰了涡虫 HSP90 的表达

为了探索涡虫再生过程中 HSP90 的可能作用，我们与指导老师及专家商量后决定利用 RNAi 技术，通过喂食 dsRNA 干扰 HSP90 表达，看涡虫再生过程是否受到影响。

3. 2. 2. 1 成功制备 HSP90 的 dsRNA

反转录扩增后，琼脂糖凝胶检测，条带大小约 650bp，与 HSP90 预期大小 658bp 相近(图 7a)。菌落 PCR 鉴定，表明 HSP90 基因成功重组到 pMD 19-T 载体上(图 7b)，测序结果也证实克隆成功。

以该重组载体为模板，从重组载体上扩增出 HSP90 基因，电泳检测其条带大小为 650bp 左右，与 HSP90 的序列长短一致(图 7c)。以此为模板，体外成功转录得到 HSP90 的 dsRNA (图 7d)。

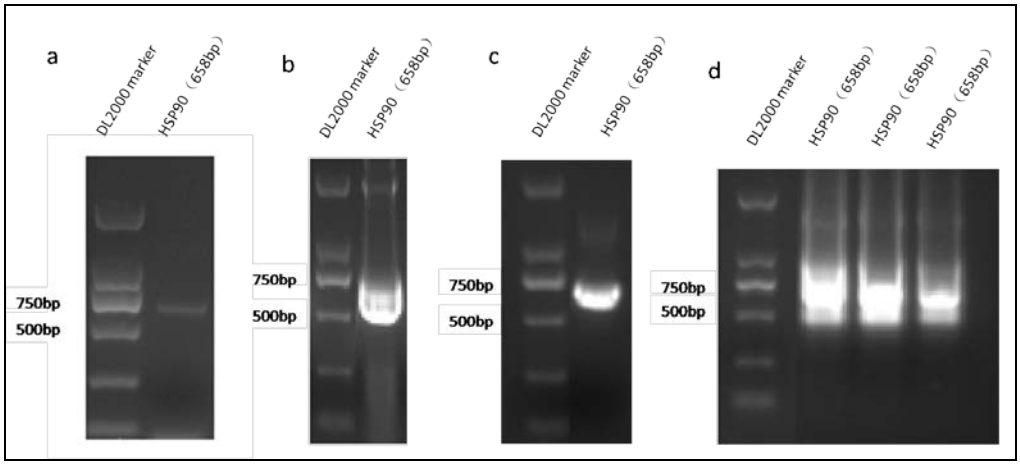

图 7. HSP90 琼脂糖凝胶电泳结果图

a 图表示从 cDNA 扩增 HSP90; b 图表示 HSP90 菌落 PCR 鉴定; c 图表示 HSP90 dsRNA 体外转录模板的扩增; d 图表示 HSP90 体外转录产物 dsRNA 电泳检测，共 3 次转录

3. 2. 2. 2 喂食 dsRNA 成功干扰了 HSP90 的表达

将体外转录获得的 HSP90 dsRNA 与涡虫食物猪肝浆混合，被涡虫摄取到体内。从图 8 可以看到，进食后的涡虫消化道泛红，说明可以通过喂食方法让涡虫把 dsRNA 摄取到体内。

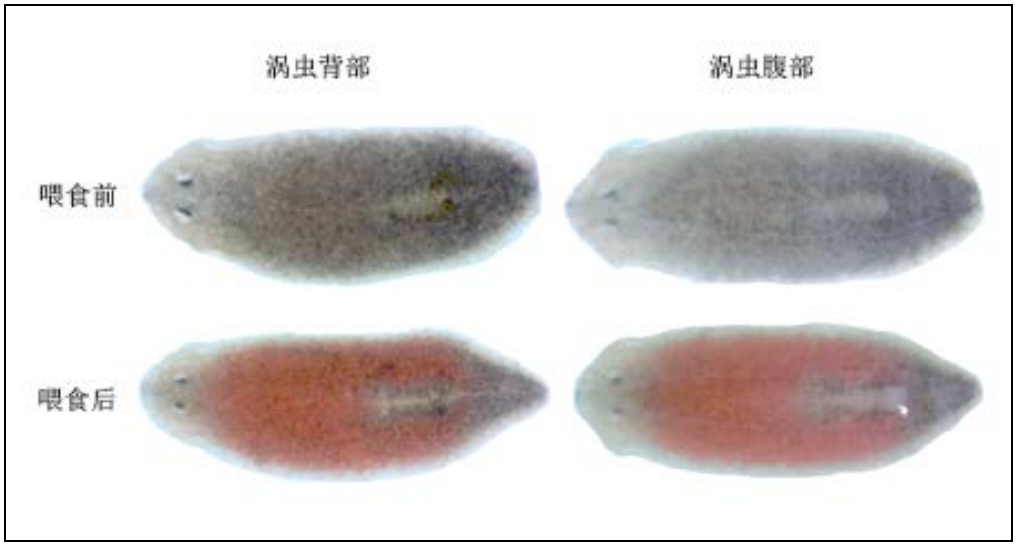

图 8. 涡虫进食前后颜色变化

摄取食用色素后变红，以直观表示涡虫摄取了食物中干扰 HSP90 的 dsRNA

喂食 dsRNA 3 次后的第 3 天，qPCR 检测 HSP90 的表达量，可以看到干扰组涡虫 HSP90 表达量明显比对照组低(图 9a)，差异极其显著(P<0.001)，说明 dsRNA 成功干扰了 HSP90 的表达。qPCR 扩增曲线(图 9b)、溶解曲线(图 9c)表明扩增特异性很好。

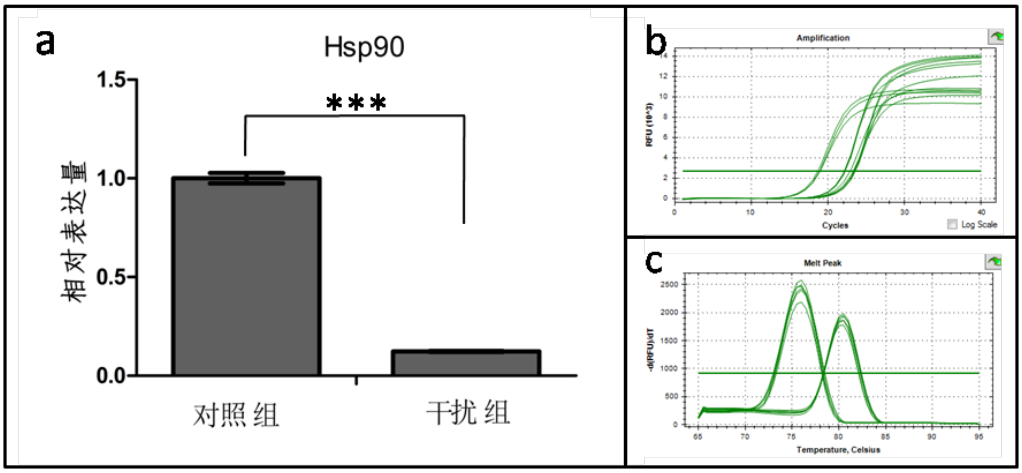

图 9. 干扰涡虫 HSP90 表达后荧光定量 PCR 结果

a. HSP90 基因被干扰后表达变化图；b. qPCR 扩增曲线图；c. qPCR 溶解曲线；***表示差异极其显著(P<0.001)

3. 2. 3 干扰 HSP90 后涡虫再生能力明显降低

成功干扰 HSP90 表达 3 天后，将涡虫在咽前切为两段，不同时间分别观察对照组与实验组涡虫头部和尾部再生情况，并拍照记录。

头部再生情况如图 10 所示。对照组涡虫再生头部过程中，再生 6h，创伤面已经收缩，再生 1 天，创伤处长出了乳白色胚基，2 天再生出眼点(图 10a“*”标注的放大图)，6 天就再生为一个完整的个体。而 HSP90 被干扰后，涡虫头部再生受到了影响(图 10b)：再生 6h，创伤面也未能应激收缩，再生 1 天，只长出了较小的胚基，再生 2 天时眼点还没有形成，直到第 3 天才再生出小小的眼点。与对照组相比，干扰组眼点再生时间延迟了 24h，且干扰组再生 6 天，也没有形成有耳突的典型头部形态。

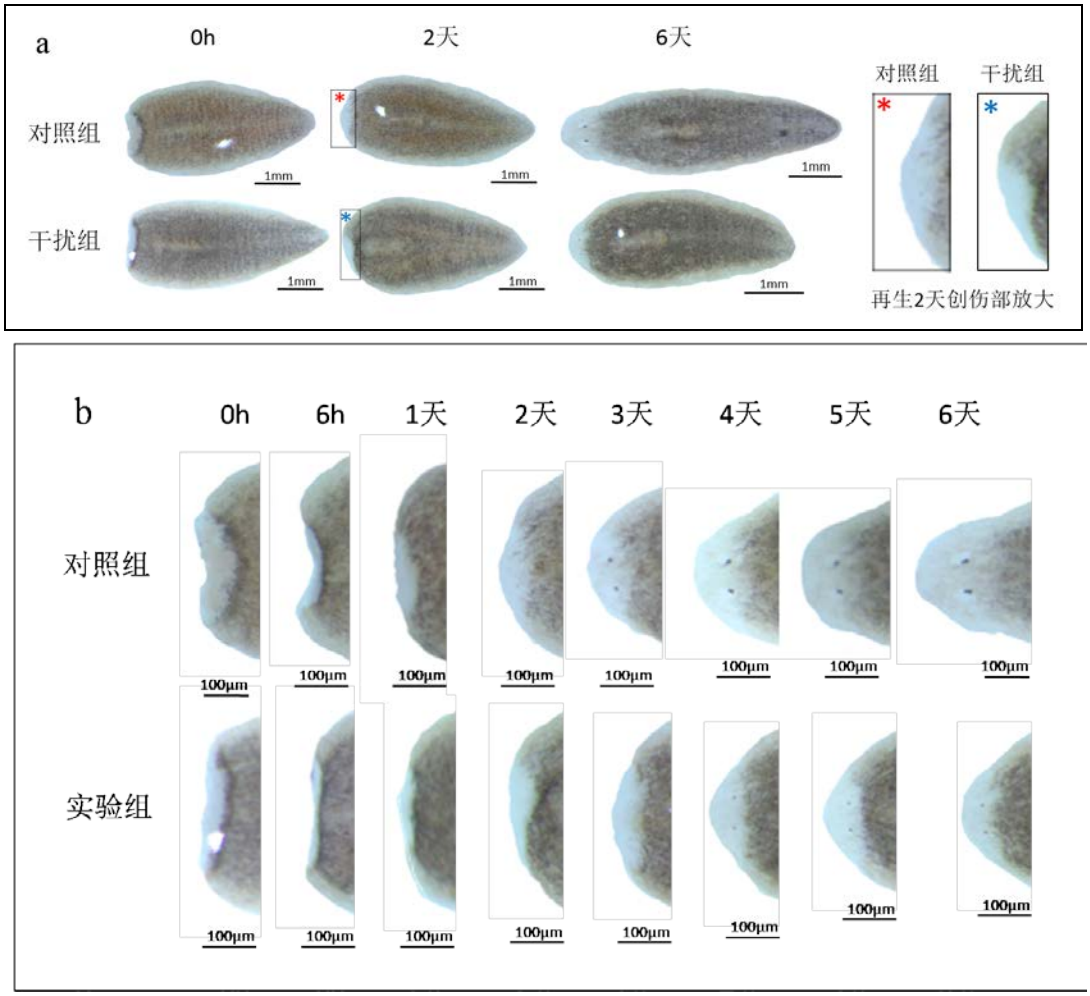

图 10. 干扰 HSP90 后涡虫头部再生过程

图 a 表示再生头部 0h、2 天、6 天情况；图 b 表示头部再生过程创伤部位放大照片

同样，尾部再生过程中(图 11)，干扰组涡虫再生 6h，创伤面也未能收缩(图 11b)，胚基形成少；再生 6 天时，也没有形成完整的个体，尾部比对照组短了 1~2mm(图 11a, b)。

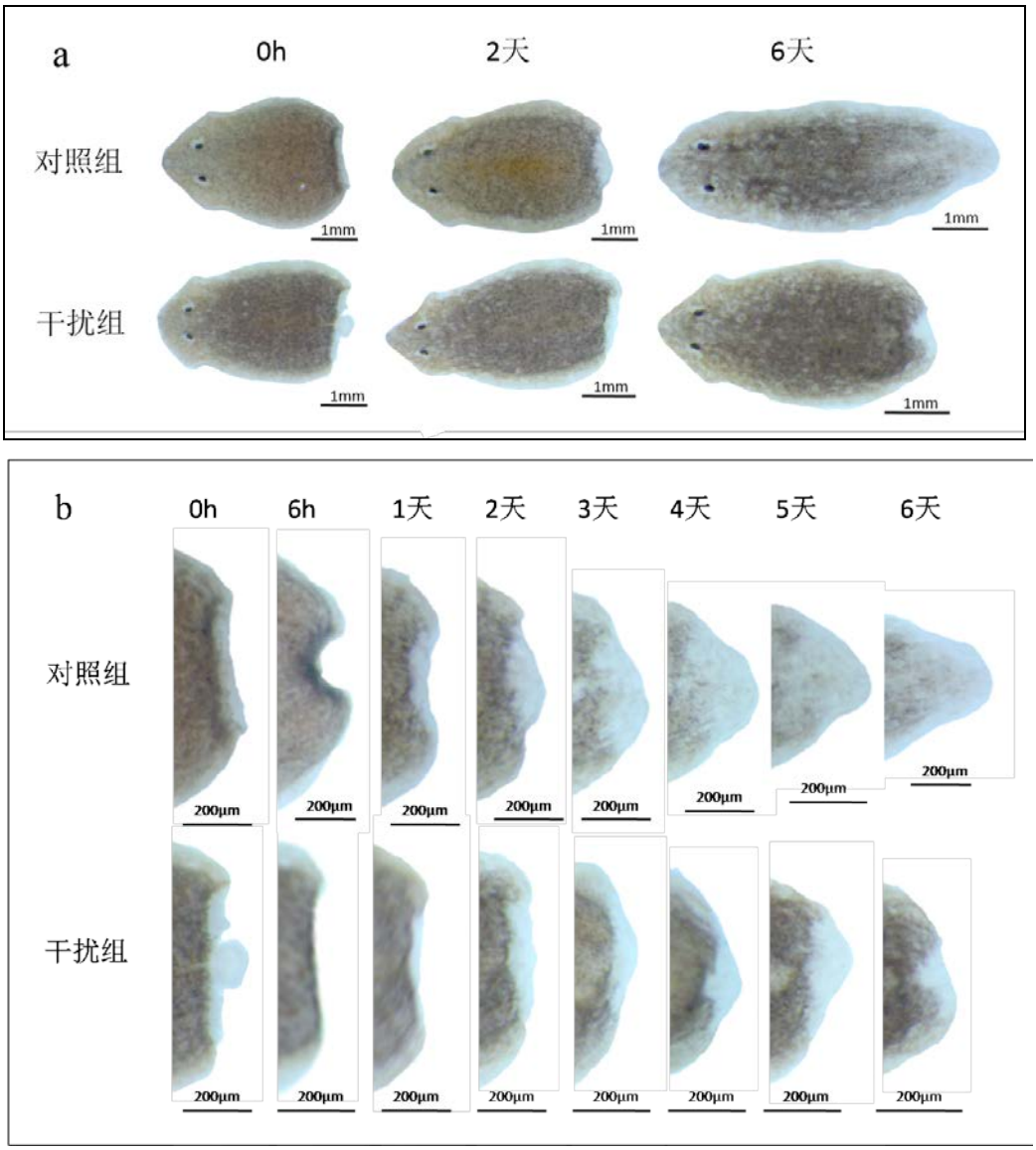

图 11. 干扰 HSP90 后涡虫尾部再生过程

图 a 表示再生尾部 0h、2 天、6 天涡虫情况；图 b 是尾部再生过程创伤部位放大照片

干扰 HSP90 后，从涡虫再生过程中长/宽比值变化也发现，涡虫再生速度减慢(表 1、图 12)。涡虫再生过程中，身体长度不断增加，原有的身体宽度会随着长度的增加而变窄，以协调新建组织和原有组织的比例以及涡虫整体的比例。涡虫身体的长度和宽度会因为运动而经常变化，为了避免涡虫蠕动，我们将载玻片放在冰上，然后将涡虫放载玻片上，这时候就是涡虫自然伸展状态。用自然伸展状态下的长/宽比值来衡量涡虫的再生速率(表 1、图 12)，发现切后再生过程中，对照组涡虫的长/宽比值不断上升，6 天后涡虫再生完成，而干扰组涡虫的长/宽比值明显低于对照组，比值平缓。这意味着干扰组涡虫再生速率极其缓慢。

表 1. 涡虫再生过程中长/宽比值

切后再生时间	对照组		干扰组	
	头部再生	尾部再生	头部再生	尾部再生
0	1.08±0.08	1.74±0.06	1.06±0.11	1.73±0.21
1 天	1.29±0.08	1.97±0.06	1.14±0.10	1.80±0.19
2 天	1.61±0.10	2.34±0.05	1.31±0.18	1.94±0.21
3 天	2.04±0.08	3.09±0.12	1.48±0.23	2.13±0.22
4 天	2.48±0.22	3.62±0.17	1.56±0.25	2.19±0.22
5 天	2.96±0.36	4.28±0.25	1.62±0.24	2.28±0.24
6 天	3.68±0.60	5.79±0.51	1.64±0.28	2.31±0.25

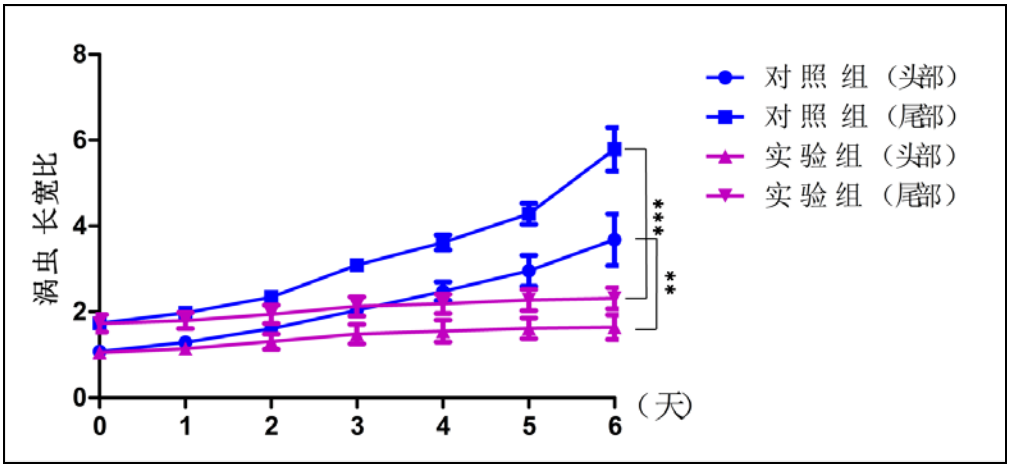

图 12. 涡虫再生过程中长/宽比值变化图

***表示 $P<0.001$, **表示 $P<0.01$

3. 3 干扰 HSP90 后影响了涡虫体内干细胞的增殖能力

我们想知道干扰组涡虫再生减慢的原因。查阅文献后，得知涡虫再生需要体内干细胞增殖分化，于是我们推测涡虫再生速度减慢可能是因为 HSP90 被干扰后，导致涡虫体内干细胞增殖能力降低。

有文章报道增殖细胞核抗原(proliferating cell nuclear antigen, PCNA)是涡虫成体干细胞增殖的一个标志基因，表达时期与 DNA 合成时期一致，而且有现成的 qPCR 引物序列(Shibata et al., 2016)。因此，检测 PCNA 的表达，可评价干细胞增殖状态。有报道涡虫切后再生第 6h 和第 3 天，PCNA 表达量会出现两次高峰(Tu et al., 2012)。于是，我们在涡虫切后 0h，6h 和 3 天，提取 RNA、反转录，利用 qPCR 检测了 PCNA 表达量的变化(图 13)。

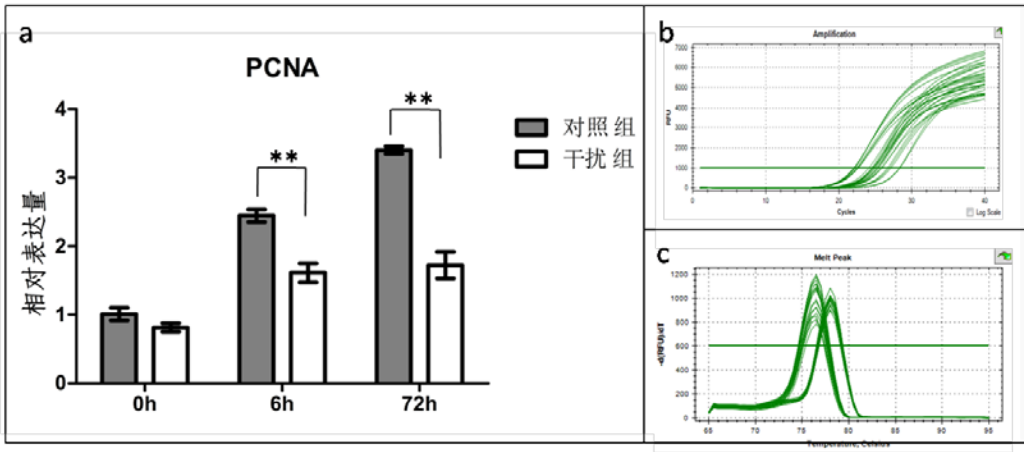

图 13. 干扰组与对照组涡虫创伤后再生 0h，6h，72h PCNA 表达量变化

a. HSP90 被干扰后 PCNA 表达变化图；b. qPCR 扩增曲线图；c. qPCR 溶解曲线；**表示差异显著(P<0.01)

从图 13 可见，尽管干扰组和对照组 PCNA 表达量在 6h 和 72h 都有升高，但干扰组涡虫干细胞 PCNA 表达量明显低于对照组(P<0.01)。这一结果更好的解释了 HSP90 在被干扰表达后，涡虫再生能力降低是因为体内干细胞增殖减少的缘故。但由于本研究中不是将 HSP90 完全敲除，只是部分干扰，所以干扰组涡虫还是能够再生，只不过再生速率明显降低了。

3. 4 HSP90 是调控涡虫神经系统创伤修复能力的关键基因

为了探究 HSP90 被干扰后，涡虫再生过程中神经系统创伤修复是否受到影响，请教专家后，找到了一种神经系统特异性蛋白的抗体 anti-SYNORF1(3C11)，并联系上了出售该抗体的公司。用这个抗体标记涡虫神经系统，免疫荧光组化实验显示涡虫神经系统的创伤修复情况。

实验结果显示，再生 0h 时，实验组与对照组相比，涡虫神经系统再生情况无明显差异(图 14a)；再生 2 天时，对照组涡虫神经系统有明显的再生，受损的神经系统前、后端再生良好，而 HSP90 干扰组涡虫受损神经系统再生迟缓(图 14b)；再生 6 天时，对照组涡虫神经系统再生完全，再生的脑神经节前端和腹神经索末端能正常闭合，而 HSP90 干扰组涡虫，头部再生 6 天，再生的脑神经节前端未能完全闭合，尾部再生 6 天，再生的腹神经索末端也没能完全闭合(图 14c)。

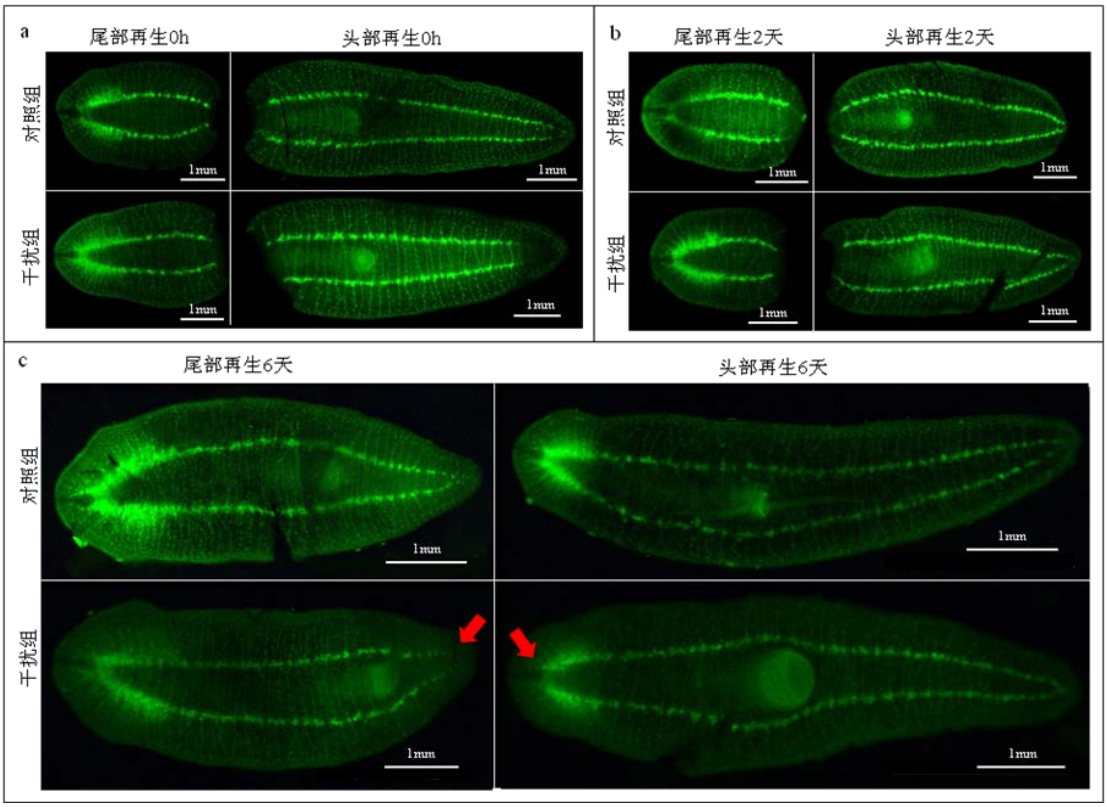

图 14. 干扰 HSP90，涡虫切后再生 2 天和 6 天时神经系统免疫荧光图

a~c 分别显示神经系统再生 0h、2 天、6 天的再生情况；红色箭头所指为干扰组未闭合的腹神经索、未闭合的脑神经节

4.讨 论

再生是自然界中非常神奇的生物学现象。为什么有些动物具备强大的再生能力，而另外一些动物的再生能力却非常有限？同样具备再生能力的蝾螈和涡虫，为什么蝾螈能再生出四肢和尾等组织器官，而涡虫哪怕被切成一小块儿也依然能够再生出完整的个体？再生的生物学机制是什么？再生的“开关”又在哪呢？这些问题存在了上百年，但因为研究技术的局限和研究模型的缺乏，再生机制一直像谜一样困扰着众多生物学家。涡虫因具备独特和惊人的再生能力而成为研究再生的重要模式生物，受到国内外很多动物学工作者的关注。近十来年，通过研究涡虫再生，找到了一些调控再生的信号通路、蛋白分子以及微小 RNA(徐振彪 等，2013)。但是，人们对涡虫再生的机制依然知之甚少。

HSP90 作为应激蛋白，介导机体在受到物理、化学或生物因子刺激时快速做出应答，参与细胞生长、发育和分化，也与肿瘤的发生发展密切相关，是一个重

要的“明星”分子。但在研究再生常用的模式动物如蠑螈或斑马鱼中，未见 HSP90 与其再生关系的报道，对于再生能力极强的涡虫，也未见报道。虽有学者成功克隆了涡虫 HSP90 基因，证实饥饿或温度升高等刺激能诱导其表达上调(马克学等，2013)，包括 HSP90 具有保护涡虫胃皮细胞的作用(程方方 等，2016)，但 HSP90 是否调控涡虫再生，仍然不得而知。

本研究体外转录 HSP90 的 dsRNA，将其与食物混合后喂食涡虫，干扰涡虫体内 HSP90 的表达，发现干扰组涡虫在切后 6h 仍不能及时做出应激反应收缩创伤面；对照组涡虫再生 2 天后，头部就再生出眼点，而干扰组涡虫在再生后的第 3 天，头部才再生出小小的眼点；从咽前切断后，对照组涡虫 6 天完成再生，而 HSP90 干扰组涡虫 6 天内并不能再生为完整个体；此外，通过比较涡虫再生不同时间长/宽比值，发现干扰组涡虫在切后虽然可以再生，但再生速率明显低于对照组。涡虫独特的再生魅力源于其体内干细胞增殖，通过检测 PCNA 的表达量，发现干扰 HSP90 后，涡虫体内干细胞增殖减少，说明干扰组涡虫再生能力的降低与其体内干细胞增殖减少密切相关。这些结果说明，干扰 HSP90 的表达后，涡虫再生能力明显降低，HSP90 是调控涡虫再生能力大小的关键基因。

神经再生是一个世界性难题。尽管涡虫神经系统在某种程度上与脊椎动物神经系统相似，具有单极、两极和多极神经细胞(胡国栋 等，2014)，但涡虫神经系统再生能力极强，能够在短短几天时间内完整地再生出有功能的神经系统。其它绝大数无脊椎动物和脊椎动物对此叹莫能及！在本实验中，我们首次发现，干扰 HSP90 后，涡虫神经系统再生修复能力降低，HSP90 是调控涡虫神经系统创伤修复能力的关键基因。

由于 HSP90 是一个在进化上高度保守的分子，加之调控涡虫干细胞增殖分化的相关基因以及信号分子在人类及其它哺乳动物中都很保守，因此我们推测，HSP90 调控涡虫再生的研究结果很可能适用于人或其它动物，比如，将 HSP90 用于受损的组织或器官，来促进创伤愈合。不仅如此，针对 HSP90，用涡虫来筛选调控神经系统再生的药物，将很有可能用于人类神经系统的损伤修复。实现人体组织或器官的再生，也许将不再是梦！

综上所述，我们可以初步得出结论，HSP90 作为应激蛋白，在涡虫创伤后的早期应激反应中发挥重要作用；干扰 HSP90 后，影响了涡虫的再生应答，致使

涡虫个体再生及神经系统创伤修复能力大大降低。因此，HSP90 是涡虫再生过程中的关键调控蛋白，并在神经系统修复中有潜在应用价值。

小 结：

- HSP90 在涡虫切后 6~12h 的创伤早期应激反应中表达量快速上升；
- 与对照组相比，干扰 HSP90 表达后，干扰组涡虫再生能力和速率明显降低，切后 6h 还不能应激收缩创伤面，眼点出现时间延迟 24h，干扰组涡虫 6 天也不能完成再生；
- 干扰组涡虫再生能力降低与其体内干细胞增殖减少密切有关；
- 干扰 HSP90 表达后，涡虫神经系统再生修复能力降低。

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每一个队员在研究报告撰写中承担的工作以及贡献:

整个项目工作是我们三个人一起共同构思、研究讨论和共同撰写完成。实验工作主要是在 2017 年暑假完成，三个同学都共同参与了全部实验，在每个实验中我们有不同的分工，以保证实验顺利进行。邹普越为项目召集人，负责整个项目的设计和实施工作。

论文撰写第一稿于 2017 年 9-12 月完成，后面又不断完善和修稿，三个人进行了不同的分工，初稿分工：项目背景部分由曹楚林完成，实验部分由邹普越完成，讨论部分由胡瀚丹完成，然后大家在一起共同反复多次修稿，包括实验数据处理、图表制作、参考文献的反复阅读，最终完成终稿！

致 谢

涡虫神奇的再生能力真的让我们惊叹和着迷，它吸引着我们进行了一段终生难忘的科学研究旅程。我们团队在这大半年的时间里不断的学习和探索，从简单的好奇到问题的提出、资料的查阅，从实验的构思和实际操作，实验数据的统计处理，到最后论文的总结写作完成，我们经历了一个辛苦而有意义的科研探索过程。在这个过程中，得到了老师和家人的大量帮助！

首先感谢指导老师——成都七中的 XX 和 XXX 老师！从最初的设想到最后论文的完成，老师对我们进行了精心指导，不断解答我们的多种疑惑，耐心教会我们多种实验技术和数据分析，为我们提供了最好的实验条件。感谢老师们陪我们走过了高中岁月中最难忘的一段学习和探索旅程！同时，成都七中 XX 老师也为我们提供了很多帮助与鼓励。

我们还遇到了不少困难、挫折与失败的打击，团队成员之间的鼓励、坚持及向老师请教，终于获得了一些研究成果。同时，我们收获了课堂上学不到的一些东西，比如培养了自己主动获取知识的意识，遇到问题想办法解决问题的能力，以及坚持不懈，遇到困难不放弃的精神，感受到了科学探索的魅力和团队合作的重要性，这些都让我们受益终生。感谢我们的努力和坚持！

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最后，要感谢我们的父母，他们至始至终不断地鼓励我们，永远都是我们成长的坚强后盾！

本参赛团队声明所提交的论文是在指导老师指导下进行的研究工作和取得的研究成果。尽本团队所知，除了文中特别加以标注和致谢中所罗列的内容以外，论文中不包含其他人已经发表或撰写过的研究成果。若有不实之处，本人愿意承担一切相关责任。

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2018 年 9 月 2 日

A new discovery of the key stress protein HSP90 in regulating the regenerative ability of the planarian

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Abstract:

Planarian is a kind of unique animal, which exhibit an extraordinary ability to regenerate lost body parts. However, its mechanism of regeneration is unclear.

HSP90 is a highly conserved stress protein. In order to explore the relationship between HSP90 and the regeneration of the planarian, several experiments were carried out in this study.

After amputation, qPCR results showed that the expression of HSP90 was obviously increased in the early planarian stress response. RNA interference by feeding in vitro-synthesized HSP90 dsRNA to planarians, and then planarians were amputated into 2 fragments in pre-pharynx at the 24th hour after the last feeding. It was found that the regenerative ability of the planarians detected in the interference group was significantly lower than that of the control group. After amputation, the wound surface could not contract at the 6th hour, the appearance of eyespots on the regenerated head was delayed 24hrs, and the planarian of the interference group did not regenerate into a complete worm after 6 days, on the contrary, the planarian of the control group regenerated into a complete individual. The regeneration rate was measured through the ratio of length and width. It was found that during the process of regeneration, the ratio of the length/width observed in the inference group was significantly lower than in the control group.

The expression of *PCNA* gene by qPCR showed that the regenerative ability of the planarian was closely related to the decrease of the stem cell proliferation in the interference group. Through immunofluorescence technique, it was found that the end of the brain ganglion and the ventral nerve cord of the interference planarian group were not completely closed on the 6th day of regeneration, while that of the control group were regenerated normally.

These results indicated that HSP90 can not only regulate the individual regeneration of the planarian, but also the healing of its nervous system, so it is the key protein in regulating the regeneration of the planarian. This study helps us to understand and explore the mechanism of the planarian regeneration and the repair of the nervous system, which has been a worldwide problem.

Key words: HSP90; planarian; regeneration; RNA-interference; nervous repair

Introduction

A biology expert was invited to give us an academic report, and the expert talked about an amazing animal called planarian, which could regenerate its head and tail after been cut off. More surprisingly, geneticist Morgan showed that a fragment as small as 1/279 of a planarian could still regenerate into a complete worm in 1898 (Morgan, 1898). Therefore, the planarian is considered to be “immortal under the edge of the knife”. The extraordinary regenerative ability of the planarian immediately aroused our curiosity: How does a tail fragment know to make a new head and a head fragment know to make a new tail? What is the secret of its regeneration? Why can't humans and many other animals regenerate like the planarian does? With these questions in mind, we searched information about planarian on Baidu and read some research papers and had a preliminary understanding about this amazing animal.

The planarian has different species which live in different environments. Freshwater water planarians belong to the Platyhelminthes and Turbellaria (Guangwen Chen, 2000), and the body is about 10mm long. The widely distributed freshwater planarians in China are *Dugesia japonica*. The body is flat and symmetrical, with the head, tail, trunk, back and the abdomen. The head is triangular. There is a pair of black eyespots on the inner side of the back. The pharynx is the in middle of the abdomen (Fig.1).

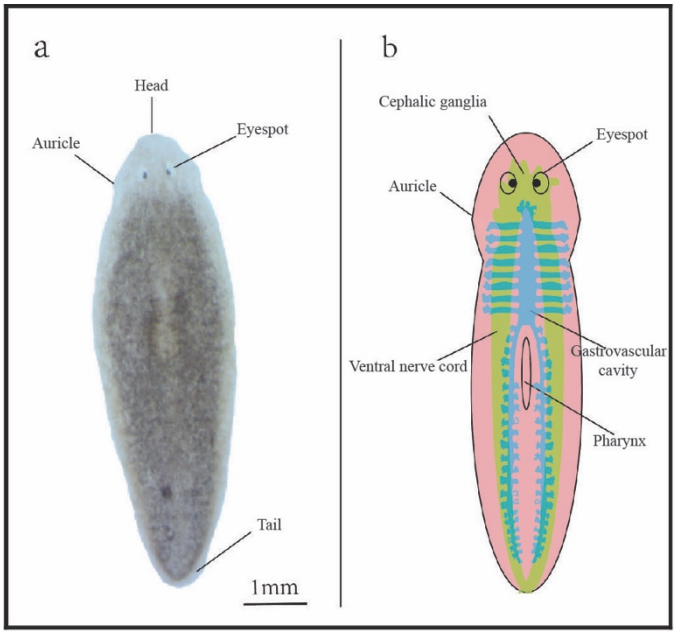

Fig. 1 The structure of the planarian

Since geneticist Morgan's research on the regeneration of the planarians, for more than 100 years, scientists have done a lot of research on the regenerative ability of the

planarian, but the early research is mainly a description about the regeneration of the planarian because of the lack of better research techniques, such as molecular biology. In recent 20 years, a number of genes and signaling pathways associated with the regeneration of the planarian have been discovered by inducing RNA interference (Newmark et al., 2002; Miki Hattori et al., 2018).

The amazing regenerative ability of the planarian is closely related to the rich adult stem cells in the body. These cells can form a complete individual by migration proliferation and differentiation. When planarian is injured, it will cause stress reaction, and then mobilize all kinds of signals to induce adults stem cells migration, proliferation and differentiation. Does wound stimulate the expression of some genes in the stem cells of the planarian, which stimulate its regeneration? We looked for some genes related to wound stress and growth, and focused an important gene Hsp90 (heat shock protein, Hsp90).

HSP90 protein is widely found in the prokaryotic and eukaryotic cells, and the structure is highly conserved because the molecular weight is about 90 kDa and is expressed in the high temperature of the body or cell, so it was named HSP90 (heat shock protein, Hsp90). Later, it was also found that, in addition to the temperature stimulation, when stimulated by other physical, chemical, or biological factors, HSP90 was also highly expressed, so it was also called stress protein (Yanguang Li, 2013). In addition, HSP90 has attracted much attention because of its involvement in cell growth, development and differentiation, tumor and fungal resistance (Liping Zhang, 2014; Qilin,Wang etc., 2011).

There were few studies on HSP90 of the planarians. It was reported to clone the HSP90 gene and analyze its expression in the environment of hot and high concentration of heavy metal and cadmium, and found that HSP90 has the role of protecting the gastric skin cells of the planarian (Kexue Ma, 2013; Fangfang Cheng, 2016). Our entire experiment was completed in August 2017 and the novelty search report was completed in January 2018. There was no report about the regulation of HSP90 on the regeneration of planarians.

Before starting this study, we proposed the hypothesis:

It is possible that the tissue deletion, as a kind of stimulation, causes the stress response of planarian after amputation, and then induces to the high expression of the

HSP90. Hence, the high expression of HSP90 is the key signal for the regeneration of the planarian. If so, what experiments can we do to prove it?

With the help of our teachers, we read papers and consulted the experts of molecular biology. Finally, we confirmed that RNA interfering HSP90 by feeding planarian is a good and easy method for us to explore the effect of HSP90 on the regulation of planarian regeneration. We designed the several experiments.

2 Materials and Methods

2.1 Materials

Planarians from a clonal strain of *Dugesia japonica* was provided by Professor XX of Tsinghua University.

DH5α competent bacteria were purchased from Qing Ke bio-company; pMD19-T vector purchased from TaKaRa company.

Chengdu No.7 high school provided: SMZ-168 with digital cameras (Motic), PCR (C1000Touch, Bio-Rad), real-time fluorescent quantitative PCR (GFX Connect, Bio-Rad), frozen centrifuge (Micro 17R, Thermo-Fisher), electrophoretic pool, etc. Sichuan University: inverted fluorescence microscope (IX73, Olympus), Other reagents and drugs are purchased in TaKaRa and other companies.

2.2 Program of Experiment

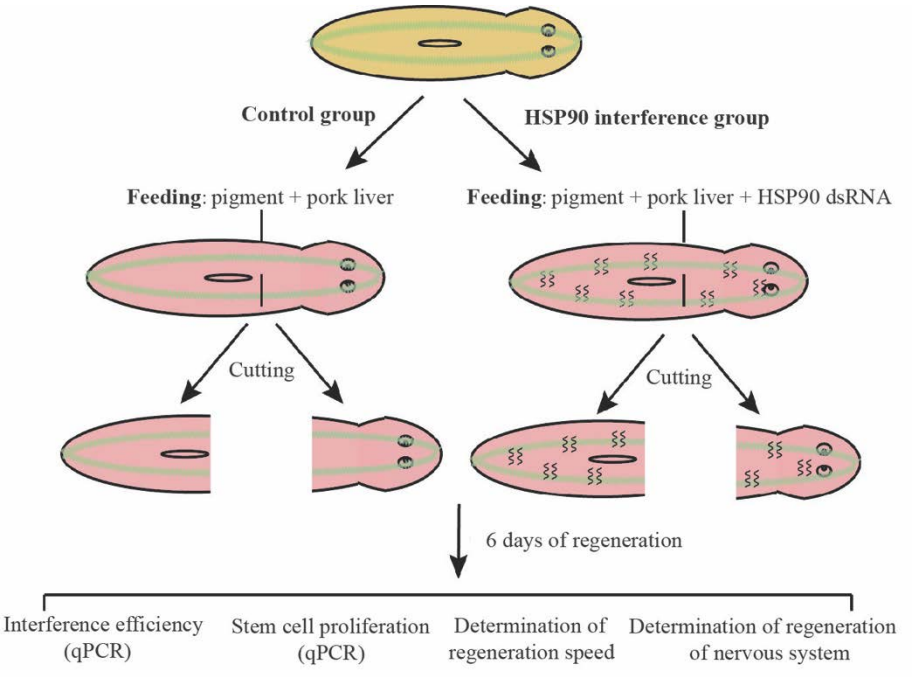

Fig. 2 The main process of the experiment (pigmentation after feeding).

It is reported that RNAi is a powerful strategy for performing loss of gene function analysis in planarians, and RNAi was first induced in planarians in 1999(Rouhana et al., 2013; Wu Xue, 2013). Therefore, we planned to synthesize HSP90 dsRNA and interfere with the expression of HSP90 by feeding dsRNA to planarians, then detected the regeneration of the planarians and the repair of nervous system by qPCR and immunofluorescence. Experimental design and main processes are illustrated as shown in Fig.2.

2. 3 Methods

2. 3.1 Feeding and amputation of planarians

Planarians were fed in distilled water at room temperature and fed with pig liver once a day for 1~2 days. Before the experiment, we selected 150 planarians, about 1cm of the length, which had been starved for 5~7 days before feeding dsRNA. In order to prevent the planarians from peristalsis, the planarians were dripped onto the glass slide, and the glass was placed on the crushed ice.

2. 3.2 Feeding dsRNA

Planarians that had been starved for at least 1 week were used in the experiments. 5 planarians per group. In the culture dish, the pig liver was scraped into puree with a knife (water: liver=1:2,V/V), and then deposited liver puree and 1% edible pigment (red) inside the 1.5 mL tube with a cut the tip of 1000 μL pipette, and mixed and centrifuged 30sec, 2,000xg, and formed into a feeding paste. Added 1μg of dsRNA per 10 μL of paste and mixed. Experiment planarians and the mixture were dripped into a Petri dish, and then the starved planarians began to eat paste about 1h in the dark environment(Fig.3).

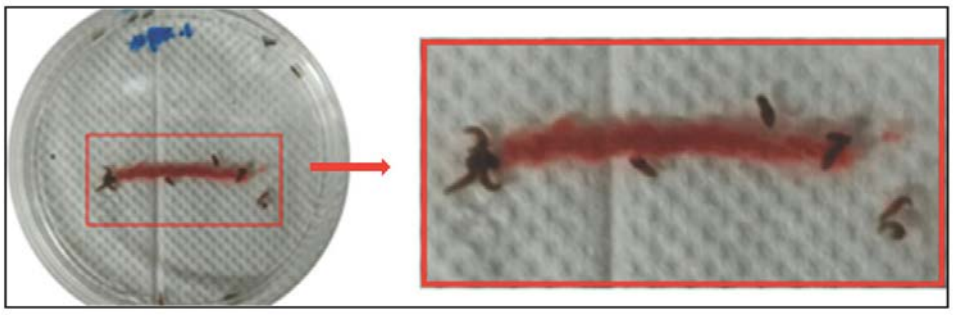

Fig. 3. Feeding method of dsRNA interference (right image is enlargement of left image and red pigmented pig liver paste).

The planarians were fed 3 times over 5 days (1^{st},$3^{rd}$and 5^{th}) (Rouhana et al., 2013).

After feeding, the planarians were placed under a microscope to see if the digestive tract became red to indicate whether dsRNA was ingested or not (Fig.3).

We fed planarians with eGFP dsRNA. qPCR showed that HSP90 expression was higher than that in water. Maybe HSP90 is sensitive to external physicochemical and biological factors. Therefore, this study did not use random dsRNA as a control. In order to make the expression of HSP90 in the control group closer to the natural, we decided to use distilled water instead of random dsRNA in the control group (Newmark et al., 2003; Almuedo-Castillo M et al., 2014).

2. 3.3 RNA Extraction and Reverse

5 planarians were placed in the 1.5mL EP tube, and 1mL reagent RNAiso plus was added. Referring to the molecular biology experiment (Wu Jianxiang et al. 2014), extract the total RNA of the planarians and reserve it and stored -80℃.

According to the reverse transcription kit instructions (TaKaRa company), the 1 μg extracted RNA was used to reverse transcription of cDNA under the condition of 37 ℃ and 85℃5S, and -80℃ stored.

2. 3. 4 DNA agarose gel electrophoresis and gel extraction

DNA agarose gel electrophoresis referred to the molecular biology experiment (Wu Jianxiang et al. 2014). DNA gel extraction method is based on the instructions of Tiangen reagent kit.

2. 3. 5 Primers design

From the National Biotechnology Information Center (NCBI) database of the US National Biotechnology Information Center (NCBI), the gene sequence was downloaded, and then the primer was designed by Primer premier 5.0.

2. 3. 6 qPCR methods

qPCR is performed as described Invitrogen manual. The levels of relative expression are calculated and quantified with the $2^{-\Delta\Delta Ct}$ method.

2. 3.7 Synthesis of HSP90 ds RNA

Due to the large amount of dsRNA needed, in order to avoid wasting cDNA, the PCR product of HSP90 was connected to the pMD 19-T carrier, and then transformed into the Escherichia coli DH5α, and selected a colony for PCR identification and sequency.After sequencing, PCR was amplified from the recombinant plasmid and extracted by gel. Then dsRNA was transcribed *in vitro* (according to the instructions of

Thermo Fisher RNA transcript Kit). The obtained dsRNA was mixed with pig liver paste to feed the worm (Fig.4).

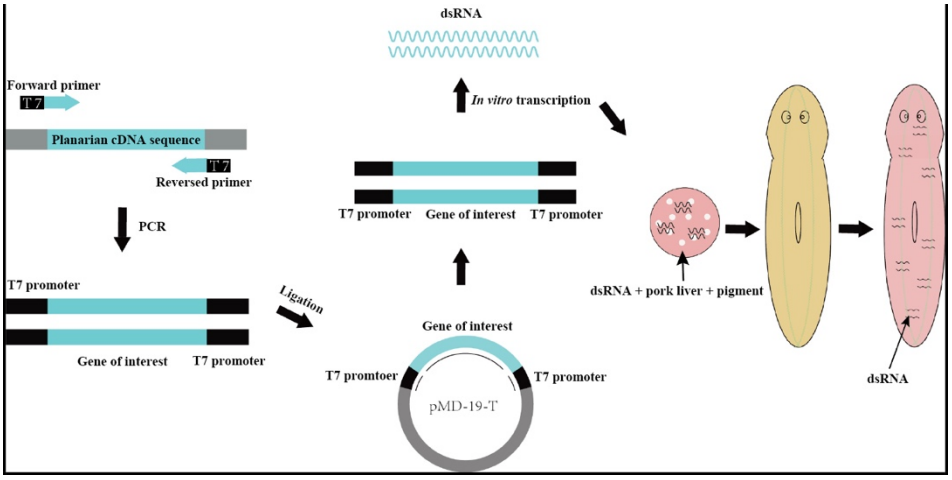

Fig.4 *In vitro* synthesis of dsRNA HSP90 and interference planarian

2. 3. 8 Measure the ratio of the planarian regeneration

The planarians were amputated into 2 fragments from pre-pharynx at the 24th hour after the last feeding, and then put their heads and tails into 6 or 12 cell plates. At 0h, 6h, 1~6 days' the planarians were photographed with a stereoscopic microscope.

Measure the ratio of the planarian regeneration: in the natural stretching state of the planarians (placed on the ice), photographed under the stereoscopic microscope, and then using Photoshop software to measure the length and width of the planarian (width measurement position: before pharynx) and calculated the ratio of length / width. The regeneration rate was described by the ratio of length to width (Tu et al. 2012).

2.3.9 Immunofluorescence analysis of nervous regeneration of planarians

After amputation, at the 0hr and on the 2nd, 6th day, the control group and the HSP90 interference group used the antibody anti-SYNORF1(3C11) of the nervous system specific marker protein (purchased in DSHB company) for immunofluorescence analysis. Finally, these planarians were observed under the fluorescent microscope. The experiment steps refer to the paper (Chen Xuhui, 2014).

3 Results

3.1 To see the powerful regenerative ability of the planarian

when we first saw the small and powerful regenerated planarians, curiosity drove us to experience the powerful regeneration of the legend planarians. The planarians were amputated in different ways to make sure if the fragment could be regenerated and repaired.

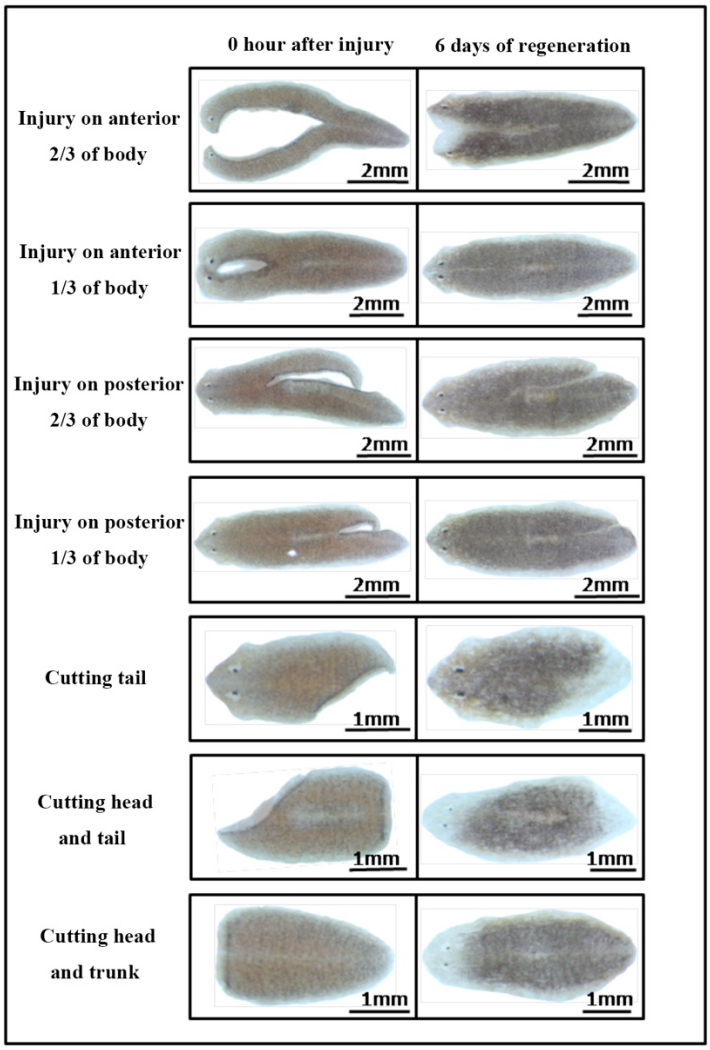

Fig.5 The map of the regeneration of the planarians after amputating in different ways

The first was to amputate the head of the planarian to a different degree of wound, the tail was retained intact; secondly, the tail end was amputated in different degrees and the head was intact. Finally, the planarian was amputated into 3 fragments, the front end was sliced, and the back end was flat, and we observed whether the middle segment really will regenerate head and tail. The results of the regeneration were observed and photographed as shown in Fig.5.

3. 2 HSP90 is the key gene in regulating the individual regeneration ability of the planarian

3. 2.1 The expression of HSP90 increased significantly after the planarians were amputated.

The HSP90 primer sequence was 5 '-TTGTCCTAAACGTGCTCCA-3', and the reverse primer sequence was 5 '-TCACGAAATTCAAATACTCTGG-3' qPCR result is

shown Fig.6a. The HSP90 expression of 6thh, 12th h of planarians increased significantly and 2 times of the control group after amputation, and the difference was very significant. The expression of 24h was as same as 0h.

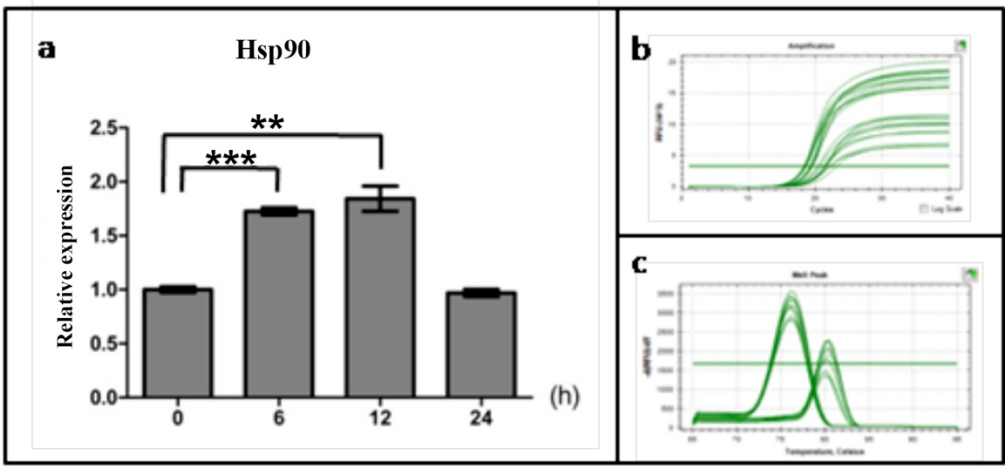

Fig.6 HSP90 qPCR at different time after amputation

a. qPCR analysis; b. the amplification curve of qPCR; c. qPCR dissolution curve; * * * indicated P<0.001, * * P<0.01

We can assume that HSP90 may play an important role in the stress response after amputation the planarians. The muscle of the planarians would shrink rapidly to reduce the area of trauma after cutting and the adult stem cells would proliferate rapidly, and the expression of HSP90 is significantly increased after cutting (Ma kexue, 2013). Thus, our inference is that HSP90 is the key protein of stress response and its increase in expression may play a vital role in the initiation of the process of planarian regeneration, so we decided to further study the role of HSP90 in the regeneration.

3. 2. 2 The preparation of HSP90 dsRNA

Reverse transcription and PCR amplification, the band of HSP90 was about 6 658bp (Fig.7a). Colony PCR identification showed that HSP90 gene was successfully recombined into pMD 19-T vector (Fig.7b), and then sequenced and confirmed that the clone was successful. The HSP90 gene was amplified from the recombinant vector by using the recombinant vector as a template. The size of the band was about 650bp, which was in accordance with the sequence length of HSP90 (Fig.7c). Using this template, the dsRNA of HSP90 was successfully transcribed in vitro (Fig. 7d).

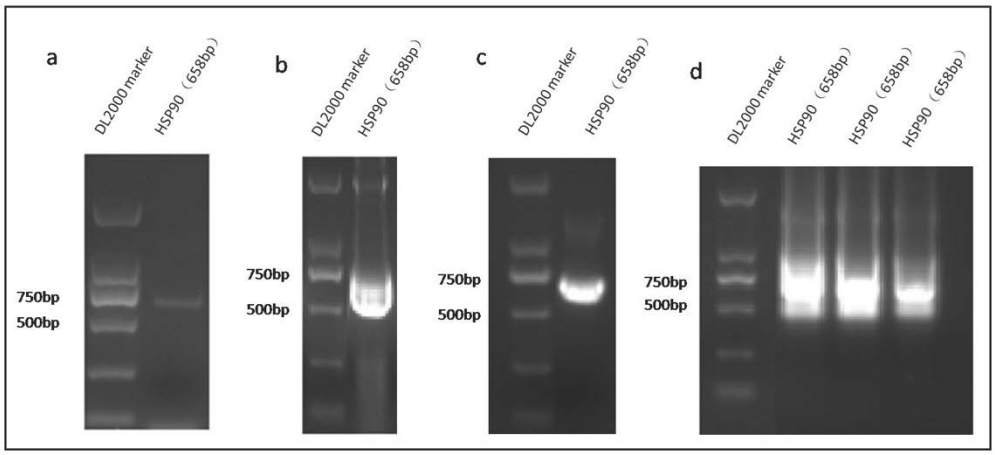

Fig 7. HSP90 agarose gel electrophoresis

a. indicated that HSP90 was amplified from cDNA; b. Colony PCR identification HSP90; c. Amplification of HSP90 dsRNA in vitro transcriptional template; d. dsRNA electrophoresis test for HSP90 in vitro transcriptional products, 3 transcripts

3. 2. 3 The successful feeding of dsRNA interfered with the expression of HSP90

HSP90 dsRNA was mixed with liver paste, and fed the planarians. The digestive tract of the planarian after eating was red, indicating that dsRNA can be absorbed into the body by feeding methods (Fig. 8).

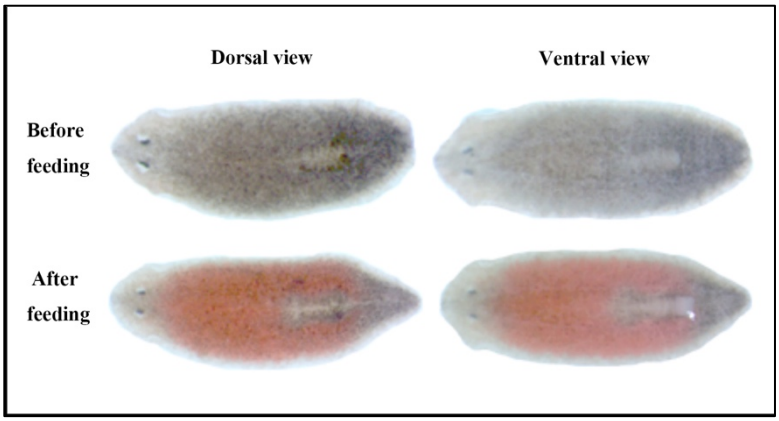

Fig.8 Color of the planarian changed after feeding of the worm. The red indicates HSP90 dsRNA have been absorbed by planarian.

We amputated planarians at the 3rd days after their third feeding dsRNA, and then detected the expression of HSP90 by qPCR. The expression of HSP90 in the interference group was significantly lower than that of the control group (Figure 9a), and the difference was extremely significant ($P<0.001$), indicating that dsRNA successfully interfered with the expression of HSP90. The qPCR amplification curve (9b) and the dissolution curve (Fig. 9c) showed that the amplification specificity was

very good.

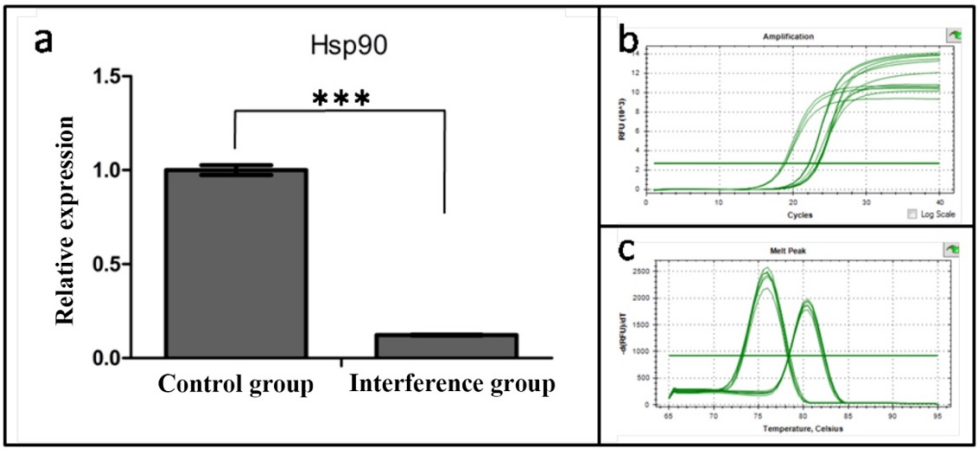

Fig. 9 qPCR after interfering with HSP90 expression in the planarians

a. HSP90 gene expression after interference; b. qPCR amplification curve; c. qPCR dissolution curve; * * * showed a very significant difference (P<0.001)

3.2.4 The regenerative ability of the planarian was significantly reduced after the HSP90 gene was interfered

After 3 days of successful interference with the expression of HSP90, the planarians were amputated into two fragments before pre-pharynx, and the head and tail regeneration of the control group and the experimental group were observed at different time, and the photos were recorded.

The head regeneration is shown in Figure 10. During the regeneration of the head of the control group, at the 6th hour the wound surface had been contracted, at the 1st day the wound was given a milky white blastema, and the eyespots appeared on the 2nd day (Figure 10a "*" enlargement of the head regeneration), and on the 6th day it was regenerated into an intact individual.

In the interference group, the regeneration of the head of the planarian was affected (Figure 10b): regenerated 6h, the wound surface failed to stress and contracted 1st day of regeneration, only a smaller blastema was grown, and the eyespots were until third days of regeneration.

Compared with the control group, the time of eyespots regeneration in the interference group was delayed 24h, and the head regeneration was completed after regenerated for 6 days.

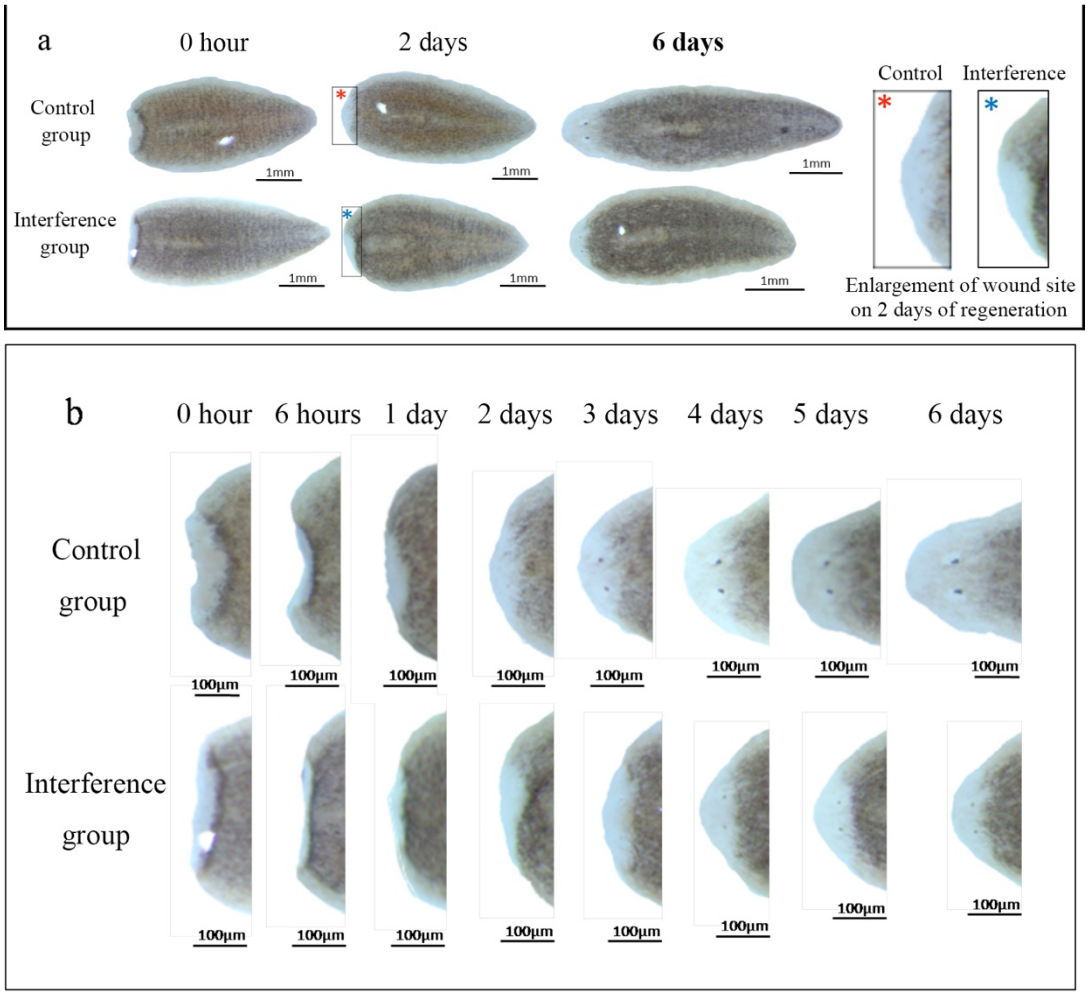

Fig.10 The head regeneration after HSP90 dsRNA interference

Fig a. shows the 0h, 2nd days, and 6th days of the regeneration head. b. shows the enlargement of the head regeneration process

During the regeneration of the tail (Fig.11), the interference group was regenerated, the wound surface did not shrink (Fig.11b), and the blastma was less, at 6h. The intact individual was not formed on the 6th day of regeneration, and the tail was 1~2mm shorter than the control group (Fig.11a).

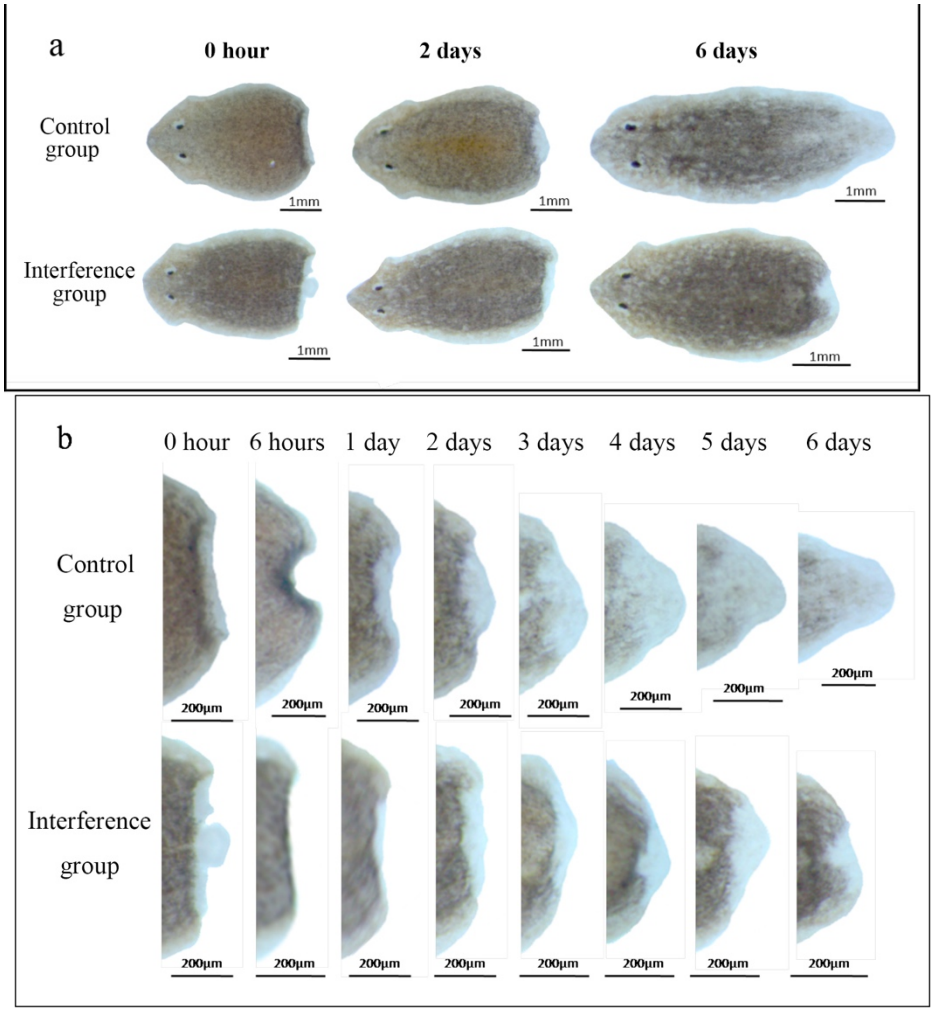

Fig.11 The tail regeneration after HSP90 dsRNA interference

Fig a. shows the situation of 0h on the regenerated tail, 2th days, and 6th days. b. is an enlarged picture of the wound during the tail regeneration process.

After interfering with HSP90, it was also found that the regeneration rate of the planarians was slowed down (Talble1 and fig12). In the process of the regeneration of the planarian, the length of the body was increasing, and the original body width will be narrowed as the length increases in order to coordinate the proportion of the new tissue and the original tissue, as well as the total proportion of the planarian.

The length and width of the body of planarian often change because of the movement. In order to avoid the planarian's peristalsis, we put the slide on the ice and then put the planarian on the slide, and then planarian was the natural extension. The ratio of the length/width of the natural extension was used to measure the rate of the regeneration of the planarian (Table 1, Fig.12). It was found that during the process of

regeneration, the ratio of the length/width of the control group increased continuously, while the ratio of the length/width of the interference group was significantly lower than that in the control group, which means that the regeneration rate of the worm in the interference group was extremely slow.

Table 1. The ratio of length / width in the process of the regeneration of the planarian

Time after cutting (days)	Control group		HSP90 interference group	
	Head regeneration	Tail regeneration	Head regeneration	Tail regeneration
0	1.08±0.08	1.74±0.06	1.06±0.11	1.73±0.21
1	1.29±0.08	1.97±0.06	1.14±0.10	1.80±0.19
2	1.61±0.10	2.34±0.05	1.31±0.18	1.94±0.21
3	2.04±0.08	3.09±0.12	1.48±0.23	2.13±0.22
4	2.48±0.22	3.62±0.17	1.56±0.25	2.19±0.22
5	2.96±0.36	4.28±0.25	1.62±0.24	2.28±0.24
6	3.68±0.60	5.79±0.51	1.64±0.28	2.31±0.25

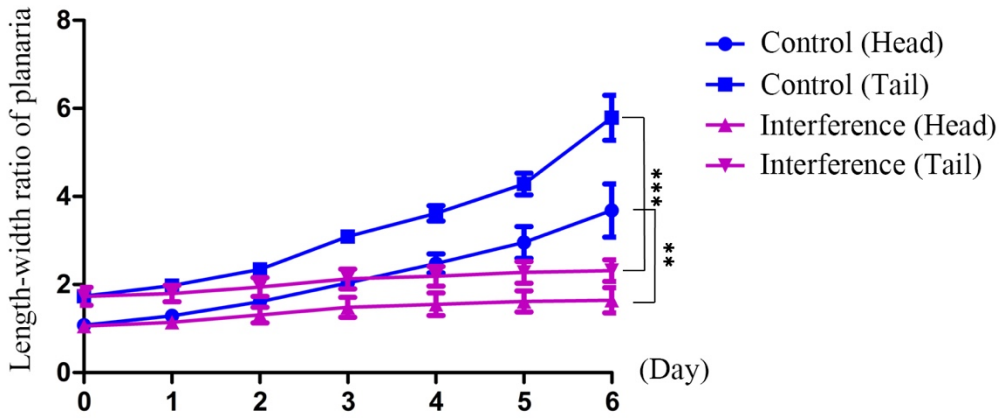

Fig.12 Length / width ratio during the regeneration of the worm

* * * P<0.001, * * P<0.01

3.3 Interference with HSP 90 affects the proliferation of stem cells in the planarian.

After looking up the papers, it was found that the regeneration of the planarian needed the proliferation and differentiation of the stem cells in the body. Therefore, we speculate that the slow regeneration of the planarian may be due to the interference of HSP90 dsRNA, which leads to a decrease in the proliferation of stem cells in the body of the planarian.

It is reported that proliferating cell nuclear antigen (PCNA) is a marker gene for the proliferation of adult stem cells in the planarian. The expression period is consistent with the period of DNA synthesis, and there is a ready sequence of qPCR primers

(Shibata et al., 216). Therefore, the detection of PCNA expression can evaluate the proliferation status of adult stem cells. It has been reported that there were two peaks in PCNA expression at the 6th hour and on the third day of the regeneration after amputation (Tu et al., 2012). We detected the expression of PCNA gene of 0h, 6h and 3rd day after amputation by PCN (Fig.13).

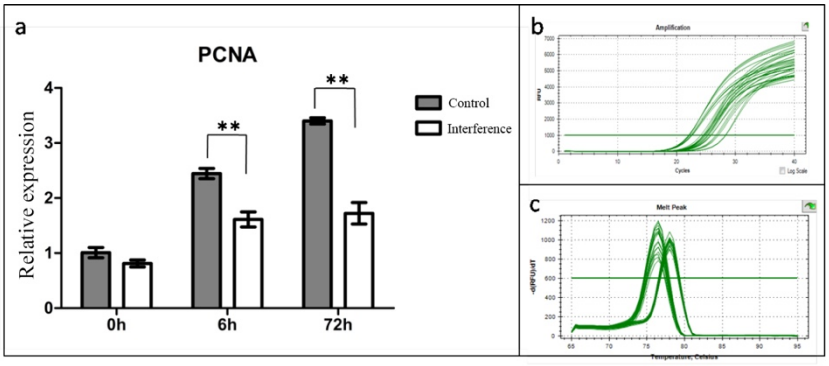

Fig.13 Expression of 0h, 6h and 72h PCNA after amputation in the interference group and control group. a. HSP90 was interfered with PCNA expression change map; b. qPCR amplification curve; c. qPCR dissolution curve; * * showed significant difference (P<0.01).

Although the expression of PCNA in the interference group and the control group increased at both 6h and 72h (Fig. 13), the PCNA expression of the stem cells in the interference group was significantly lower than that of the control group (P<0.01). This result better explained that after HSP90 gene was interfered, the regenerative ability of the planarian was reduced because of the decrease of stem cell proliferation. However, in our study, HSP90 is not completely knocked out, only partial interference, so the interference group can still be regenerated, only the rate of regeneration is significantly reduced.

3.4 HSP90 is the key gene for regulating the wound repair ability of the nervous system of the planarian

We found an antibody anti-SYNORF1 (3C11), which can mark specific protein of the nervous system of the planarian. Immunofluorescence showed the healing of the nervous wound of the planarian.

The results showed that, at 0h after amputation, there was no significant difference in the regeneration of the nervous system in the interference group compared with the control group (Fig 14a). At the 2nd day after amputation, the anterior and posterior ends of the damaged nervous system were well regenerated in the control group, while the HSP90 interference group was delayed (Fig 14b). After 6 days of regeneration, the

nervous system of the planarian of the control group was regenerated completely, the end of the brain ganglion and the ventral nerve cord could be closed normally, while the brain ganglion and tail ventral nerve cord of the HSP90 interference group were not completely closed (Fig.14c).

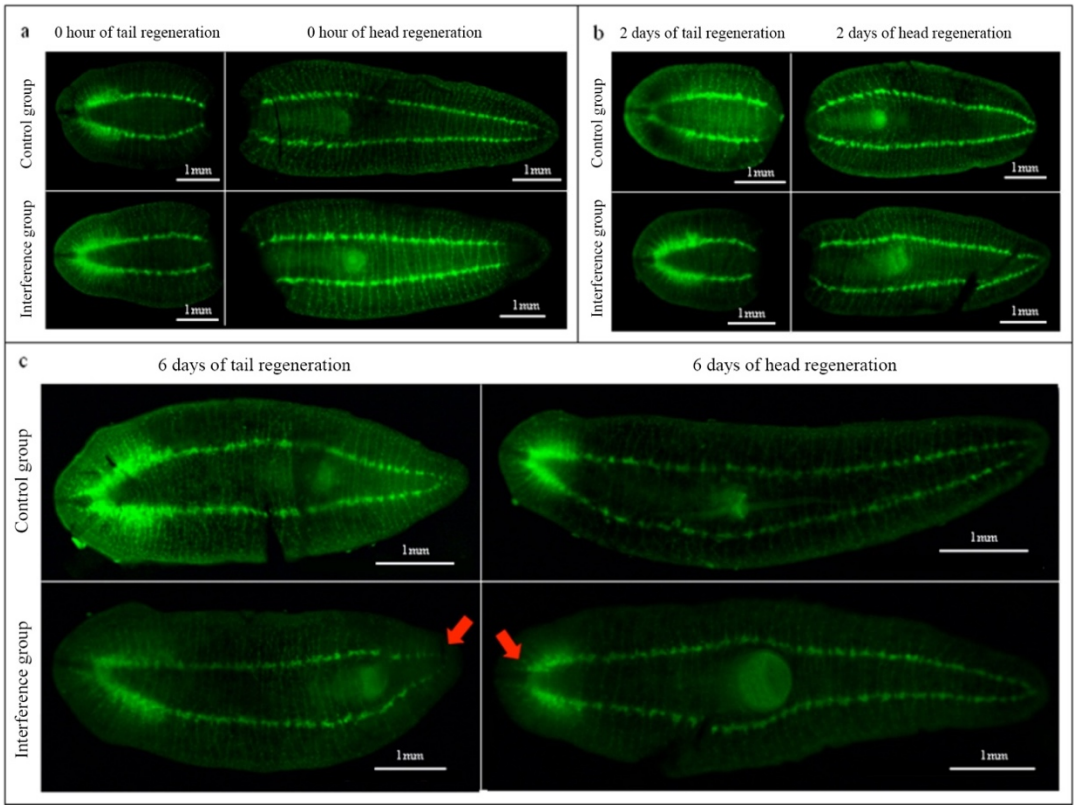

Fig. 14. Immunofluorescence of nervous system after interference with HSP90 and 2 days and 6 days after amputation. a~c showed regenerative of 0h, 2 days and 6 days of nerve regeneration respectively.

4. Discussion

Regeneration is a very mysterious biological phenomenon in nature. Why do some animals possess strong regenerative ability, while others have very limited regeneration capacity? Although the salamanders and planarians have the ability to regenerate, salamanders can only regenerate limbs and tail tissues, and planarians can still regenerate into a complete individual even if it is being cut into a tiny piece? What is the biological mechanism of regeneration? Where is the regenerative "switch"? These problems have existed for hundreds of years, however, because of the limitations of research technology and the lack of research models, the regeneration mechanism has puzzled many biologists. Because of its unique and amazing regenerative ability, it has

become an important model organism for research regeneration, and has attracted the attention of many zoologists in the world. In recent ten years, we have found some signal pathways, protein molecules and tiny RNA (Xu Zhenbiao et al. 2013) to regulate regeneration. However, little is known about the mechanism of the regeneration of the planarian.

HSP90, as a stress protein, mediates the rapid response of the body to the stimulation of physical, chemical or biological factors. It participates in cell growth, development and differentiation. It is also closely related to the development of tumor. It is an important "Star" molecule. However, there is no report on the relationship between HSP90 and its regeneration in these model animals, such as planarian, salamander or zebra fish, which are commonly used in the study of regeneration. Although some scholars have successfully cloned the HSP90 gene of the planarian, it is proved that starvation or higher temperature can induce its up-regulated expression (Marx, 2013), including the role of HSP90 to protect the gastric skin cells of the planarian (Fang Fang, 2016), but it is still unknown whether HSP90 regulates the regeneration of the planarian.

In this study, dsRNA of HSP90 was transcribed in vitro, and it was mixed with food to feed the planarian and interfered with the expression of HSP90. It was found that the inference group was still unable to respond to the stress response and the wound surface can't contract at the 6^{th} hour after the amputation. The head eyespots were appeared in the control group after 2 days of regeneration, while the head was at 3 days after regeneration in the inference group. The planarian of the inference group could not regenerate into an intact worm while that of control group could do so in 6 days after amputation from pre-pharynx. In addition, by comparing the ratio of length/width at the different time of the regeneration of the planarian, it was found that although the interference group could regenerate, the rate of regeneration was obviously lower than that of the control group. The unique regeneration charm is due to the proliferation of stem cells in the planarian body. By detecting the expression of PCNA, it was found that the decrease of the regeneration capacity of the interference group is closely related to the decrease of the proliferation of stem cells. These results indicated that HSP90 is the key gene in regulating the regeneration capacity of the planarian.

Nervous system regeneration is a worldwide problem. Although the nervous system of the planarian is similar to the nervous system of vertebrates to some extent, it has monopole, bipolar and multipolar nerve cells (Hu Guodong, 2014), but the regenerative

ability of the nervous system is powerfully strong. It can regenerate the functional nervous system in a few days, while other invertebrates and vertebrates cannot. In this experiment, we have discovered for the first time that after the interference of HSP90, the ability of regeneration and the reparation of the nervous system of the planarian was reduced, and HSP90 is the key gene for regulating the ability of wound repair of the nervous system of the planarian.

Since HSP90 is a highly conserved molecule in evolution, genes and signal molecules regulating the proliferation and differentiation of the stem cells of the planarian are very conservative in both human and other mammals. We speculate that the results of HSP90 regulation of the regeneration of the planarian are likely to be applicable to human or other animals. HSP90 promote the repair of damaged tissue or organs. Not only that, for HSP90, screening of drugs regulating the regeneration of nervous system by using the planarian will probably be used for the repair of human nervous system. The regeneration of human tissues or organs may no longer be a dream!

In summary, we can draw a conclusion that HSP90, as stress protein, plays an important role in the early stress response after the wound of the planarian. After the interference of HSP90, the regeneration response of the planarian was affected, resulting in the reduction of regeneration and nervous system wound healing ability. Therefore, HSP90 is a key regulatory protein in the process of regeneration of the planarian, which has the potential to being applied in the repair of the nervous system.

Conclusion:

1. The expression of HSP90 increased rapidly in the early stress response of 6~12h after amputation.
2. Compared with the control group, after interfering HSP90 expression, the regenerative ability and the rate of regeneration of the planarian of the inference group were significantly reduced. After amputation, the contraction of the wound surface could not stress at the 6^{th} hour, and the appearance of the eyespots was delayed 24hrs, and the planarian of the interference group could not regenerate into an intact individual.
3. The reduction of regenerative ability of the planarian of the inference group was closely related to the decrease of stem cell proliferation.
4. After interfering with HSP90 expression, the ability of regeneration and the repair of the nervous system of the planarian was reduced.

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