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Rspo1调控斑马鱼胚胎汇聚延伸运动的研究

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摘要: Rspo1 (R-spondin 1)是分泌型Rspos (R-spondins)蛋白家族的成员, 在雌性发育、血管生成和癌症等多个方面具有调控作用。为了研究Rspo1在早期胚胎发育中的功能, 以斑马鱼(*Danio rerio*)作为模式生物, 利用反转录PCR及原位杂交技术检测*rspo1*基因的时空表达模式; 通过显微注射*rspo1* mRNA或*rspo1*反义寡核苷酸(Morpholino, MO)对*rspo1*进行过表达或敲降; 通过形态观察及原位杂交技术检测胚胎汇聚延伸(Convergence and extension, CE)运动是否正常; 利用荧光素酶活性检测实验测定Wnt/PCP信号通路活性水平; 通过蛋白印迹法检测表征Wnt/PCP信号通路活性的磷酸化JNK (Jun N-terminal kinase)蛋白的水平。结果显示: *rspo1*为母源基因, 在12hpf前胚胎中呈全身性表达, *rspo1*的过表达或敲降均影响胚胎的CE运动; 过表达*rspo1*降低Wnt/PCP信号通路报告质粒的活性, 而敲降*rspo1*则增加其活性, 与之相一致, *rspo1*敲降的胚胎中磷酸化JNK的水平显著升高; 此外, *rspo1* mRNA与*dnJNK* (Dominant negative JNK) mRNA的共同注射, 以及*rspo1* MOs与*wnt11*或*wnt5b* mRNA的共同注射均能协同诱导CE运动缺陷。综上, 在斑马鱼胚胎中, Rspo1通过负调控Wnt/PCP信号通路来调控胚胎的汇聚延伸运动。

关键词: Rspo1; 汇聚延伸运动; Wnt/PCP信号通路; 斑马鱼

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在脊椎动物胚胎中, 原肠化运动是驱动胚层形成及体轴形成的形态发生运动。原肠化运动中的汇聚延伸(Convergence and extension, CE)运动在胚体“塑形”过程中发挥着非常重要的作用。CE运动是形态发生的主要过程, 该过程中胚层在中外侧方向变窄, 同时沿着背部中线在前后端方向延伸[1—5]。胚胎通过CE运动实现体轴的建立, 在脊椎动物中CE运动的详细分子机制还有待深入研究。目前已知非经典的Wnt/PCP (Planar cell polarity)信号通路是调控CE运动的关键信号[6—11]。Wnt/PCP信号通路可以通过Wnt配体的非经典家族成员(包括Wnt4、Wnt5a和Wnt11)与Frizzled受体之间的相互作用而被激活, 然后由一些下游成分如小分子GTP酶RhoA和Rac、Jun N端激酶(Jun N-terminal kinase, JNK)介导, 进而调节肌动蛋白聚合和细胞骨架动力学[12—17]。

Rspo1 (R-spondin 1)是分泌型Rspos (R-spondins)蛋白家族的成员, 它能协同Wnt配体增加Wnt/β-catenin信号通路的活性[18, 19]。人(*Homo sapiens*) *RSPO1*基因突变会导致一种隐性综合征, 其特征是XX性别逆转, 并伴有掌跖角化病, 易于罹患鳞状细胞癌[20]。在小鼠(*Mus musculus*)中敲除*Rspo1*会影响卵巢的分化, 提示*Rspo1*在雌性发育中发挥重要作用[21, 22]。在斑马鱼(*Danio rerio*)中研究发现, Rspo1-Wnt调控信号能促进血管生成, 且Rspo1通过激活Wnt/β-catenin信号通路特化造血干细胞[23, 24]。最近, 有报道表明Rspo1/RSPO1具有非依赖Wnt/β-catenin的作用, 它能通过Rspo1-Lgr4-cAMP-ERα轴调节雌激素受体的表达, 并且在结肠癌中RSPO1/LGR5能介导TGFβ信号的激活[25, 26]。鉴于Rspo1多方面的调控作用, 我们推测Rspo1参与了胚胎早期的发育。

本研究利用模式动物斑马鱼, 通过过表达和敲降实验揭示了Rspo1对胚胎早期汇聚延伸运动的调

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控作用, 并对其作用机制进行了初步探索。

1　材料与方法

1.1　主要试剂

KOD DNA聚合酶购于Toyobo公司, 限制性内切酶购于New England BioLabs公司, TRIzol购于Invitrogen公司; M-MLV及Dual-Luciferase Assay Kit购于Promega公司, DIG RNA labeling mix购于Roche公司, mMESSAGE mMACHINE Kit购于Ambion公司, 磷酸化SAPK/JNK抗体(货号9251)购于Cell Signaling Technology公司, SAPK/JNK抗体(货号SC-571)及β-Catenin抗体(货号SC-7199)购于Santa Cruz Biotechnology公司, 反义寡核苷酸(Morpholino, MO)由Gene-Tool公司设计合成。

1.2　斑马鱼品系

本研究以野生型斑马鱼Tübingen品系为研究对象。通过自然交配获得受精卵, 置于胚胎培养液中, 28.5℃生化培养箱中培养, 胚胎分期根据发育阶段划分标准进行[27]。动物实验操作采用三卡因麻醉, 并尽量将痛苦降至最低。所有实验均符合中国海洋大学的实验动物指导要求。

1.3　反转录PCR (RT-PCR)及整胚原位杂交(WISH)

用TRIzol试剂提取斑马鱼胚胎的总RNA, 用M-MLV反转录成第一链cDNA。用含有*rspo1* 3′UTR (Untranslated region)的质粒及DIG RNA labeling mix按照标准步骤制备正义和反义RNA探针。PCR引物序列如下: *rspo1*-RT-F: 5′-CCCCGACTCTTCATCTTACTG-3′; *rspo1*-RT-R: 5′-TTCTTGTTTCCTCCTCTTTCC-3′; *β-actin*-RT-F: 5′-CTTGCGGTATCCACGAGAC-3′; *β-actin*-RT-R: 5′-GCGCCATACAGAGCAGAA-3′; *rspo1*-probe-F: 5′-GAACTAAGATGCTGGCTCG-3′; *rspo1*-probe-R: 5′-CCGTAACAGTCCGTCAAT-3′。

1.4　Capped mRNA的合成、MO的合成及显微注射

Capped mRNA根据说明书用mMESSAGE mMACHINE Kit合成。为了敲降*rspo1*, 由Gene-Tool公司专业人员设计合成两个互不重叠的特异封闭*rspo1*基因翻译的反义MOs, 并如报道所述方法稀释[28, 29]。构建翻译抑制型MOs有效性检测特异报告质粒, 即*rspo1* 5′-UTR-GFP。将包含*rspo1* MO靶点的5′UTR序列(–200— –1碱基序列)和部分ORF序列(ATG起始, 1—316碱基序列), 即–200—316碱基序列, 连接至pCS2-eGFP表达载体, 用于检测MOs的有效性。将稀释的mRNA和/或MOs和/或DNA质粒注射到1细胞期的斑马鱼胚胎中。

1.5　荧光素酶活性检测

体内荧光素酶检测方法如报道所述[28, 29]。在1细胞期的胚胎中注射100 pg AP-1报告质粒和20 pg Renilla内参报告质粒, 与MOs和/或mRNA进行共注射。待胚胎发育至90%下包时期, 每组设两个平行, 分别收集15枚以上胚胎进行裂解。使用Dual-Luciferase Assay Kit进行荧光素酶活性检测。以Renilla荧光素酶活性为内参计算AP-1报告质粒活性相对值。

1.6　蛋白印迹(Western Blot)

斑马鱼胚胎发育至所需时期时, 去除卵膜, 每3条鱼加入1 μL RIPA裂解液(50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 0.5%脱氧胆酸钠, 蛋白酶抑制剂及磷酸酶抑制剂现用现加)研磨裂解, 将裂解液12000×*g*离心, 保留上清液。每个样本都用相应的抗体进行SDS-PAGE蛋白印迹。

1.7　数据统计分析

使用GraphPad Prism version 5.01软件进行数据统计分析。所有统计数据以means±SE表示, 组间差异用*t*-Test方法进行分析, 当$P<0.05$时认为有显著性差异。

2　结果

2.1　敲降或过表达斑马鱼*rspo1*基因影响胚胎汇聚延伸运动

为了研究Rspo1蛋白在斑马鱼胚胎中的功能, 我们通过参考斑马鱼基因组数据库, 克隆了斑马鱼*rspo1*基因。斑马鱼Rspo1是分泌型Rspos家族的成员, 它含有一个信号肽(Signal peptide, SP)结构域、2个FU (Furin-like)结构域FU1和FU2及1个TSP1 (Thrombospondin type I repeat)结构域(图 1A)。首先检测斑马鱼*rspo1*的时空表达模式。RT-PCR结果表明, 斑马鱼*rspo1*是母源性基因, 在受精后小时数(Hours post fertilization, hpf)为0—4时即能检测到表达, 且表达量较高。*rspo1*的表达在6—24hpf时下降, 但从36hpf开始升高(图 1B)。斑马鱼*rspo1*反义探针的原位杂交结果表明, 在1细胞期到12hpf早期胚胎中, *rspo1*呈全身性表达模式(图 1C)。*rspo1*的普遍表达和动态表达表明, 斑马鱼Rspo1可能在胚胎发育调控中发挥重要作用。

为了研究*rspo1*基因在斑马鱼早期胚胎发育中的作用, 我们首先进行了体内过表达实验。在1细胞期注射*rspo1*的capped mRNA, 待胚胎发育至12hpf进行观察。与未注射组(WT)及注射对照组(*gfp*)相比, *rspo1*注射组胚胎出现明显的CE运动缺陷, 胚胎头尾端未能延伸到正常位置, 二者之间夹角变大(图 2A和2B)。为了进一步研究*rspo1*在胚胎

发育中的作用, 我们用两个互不重叠的、特异靶向*rspo1* 5′UTR的反义MOs, 即*rspo1* MO1 (靶向–54—–30区域)和MO2 (靶向–21—4区域), 阻断内源*rspo1* mRNA翻译进程, 可抑制母源mRNA和合子型mRNA的翻译, 用以在斑马鱼胚胎中进行基因敲降实验(图 2C)。通过将MOs (MO1和MO2)与*rspo1* 5′-UTR-GFP表达载体在斑马鱼胚胎共注射来评价MOs靶向*rspo1*的效率, 我们发现MO1和MO2都能成功地封闭检测质粒的GFP标签的表达(图 2D)。随后, 将MO1 (8 ng)或MO2 (6 ng)在1细胞期注射到斑马鱼胚胎。与空白对照组(WT)及注射对照组(cMO)相比, MO1和MO2注射组胚胎在12hpf时期均表现出CE运动缺陷, 前后端夹角变大(图 2E和2F)。将低剂量的*rspo1* mRNA与MO1或MO2共同注射, 能营救MO单独注射所诱导的CE运动缺陷表型, 这表明MOs敲降作用是特异的(图 2F)。

研究表明Rspo1可以增强Wnt/β-catenin信号通路, 而Wnt/β-catenin信号通路对背腹轴的形成起关键作用[30—32]。背腹轴形成可能会影响CE运动[31], 为揭示*rspo1*过表达或敲降引起的CE运动缺陷是否与早期胚胎背腹轴分化有关, 我们用WISH方法检测了敲降*rspo1*的6hpf胚胎中背腹轴标记基因的表达模式。背侧标记基因*chd* (*chordin*)和腹侧标记基因*eve1* (*even-skipped-1*)在*rspo1*敲降胚胎中表达模式与对照组相比无明显差异, 表明*rspo1*敲降导致的胚胎CE运动缺陷不是由于背腹轴的改变引起的(图 2G)。随后进一步检测了与胚胎CE运动相关的标记基因, 神经外胚层边缘区域标记基因*dlx3b* (distal-less homeobox 3b)、脊索前板标记基因*hgg* (hatching gland)和脊索标记基因*ntl* (no tail), 以探究*rspo1*基因敲降对胚胎CE运动的影响。如图 2H所示, 与WT和cMO对照组胚胎相比, *dlx3b*、*ntl*和*hgg*的表达模式在*rspo1*敲降胚胎中发生了改变。对照组*hgg*的位置位于*dlx3b* (垂直线)前面, *dlx3b*呈中等宽度(水平线), *ntl*呈后部细表达(星号), 而在*rspo1*敲降胚胎中, 脊索前板(*hgg*)后移、神经板(*dlx3b*)变宽、脊索(*ntl*)变粗。上述研究结果表明过表达和敲降*rspo1*均会导致胚胎CE运动缺陷。已知一些CE调控基因的功能-获得或功能-缺失都会影响原肠化运动, 这些结果表明Rspo1是斑马鱼原肠

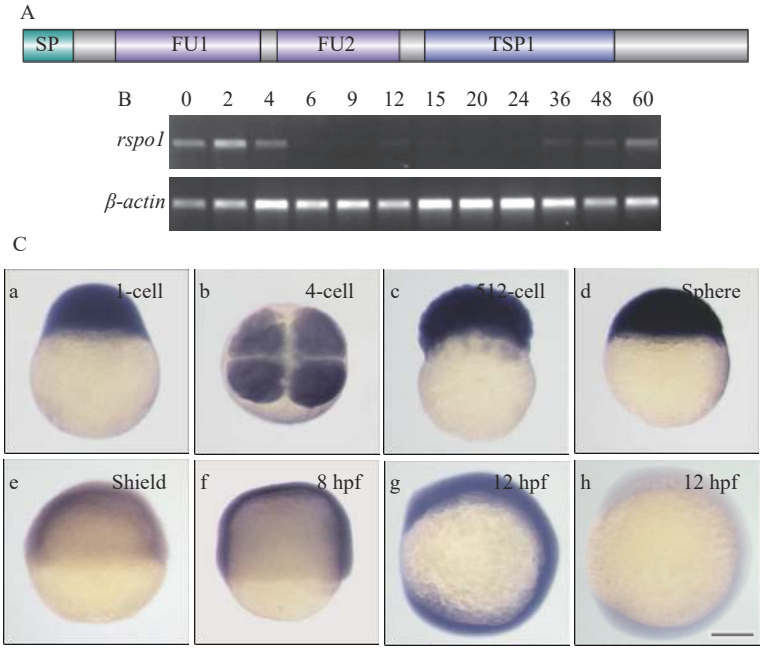

图 1 斑马鱼Rspo1蛋白结构域示意图及mRNA时空表达谱

Fig. 1 Zebrafish Rspo1 protein structure and mRNA spatiotemporal expression pattern

A. 斑马鱼Rspo1蛋白结构示意图。SP. 信号肽结构域; FU. furin-like结构域; TSP1. thrombospondin type I repeat结构域; B. 不同发育时期的半定量 RT-PCR。数字表示不同发育时期, 以受精后小时数(hpf)表示。*β-actin*作为内参基因; C. 不同发育时期原位杂交。a、c、d、e、f为动物极向上的侧面观; b为动物极向上的俯视图; g和h为动物极向上前端朝左的侧面图。标尺: 200 μm

A. Schematic structure of the zebrafish Rspo1 protein. SP. signal peptide domain; FU. furin-like domain; TSP1. thrombospondin type I repeat domain; B. RT-PCR analysis of zebrafish *rspo1* mRNA at the indicated embryonic stages. Numbers represent different developmental stages by hours post fertilization (hpf). *β-actin* as internal control; C. Whole-mount *in situ* hybridization analysis of zebrafish *rpso1* mRNA at the indicated stages. a, c, d, e and f panels are lateral views with animal pole up; b panel is top view with animal pole up; g and h are lateral view with animal pole up and anterior to the left. Scale bar: 200 μm

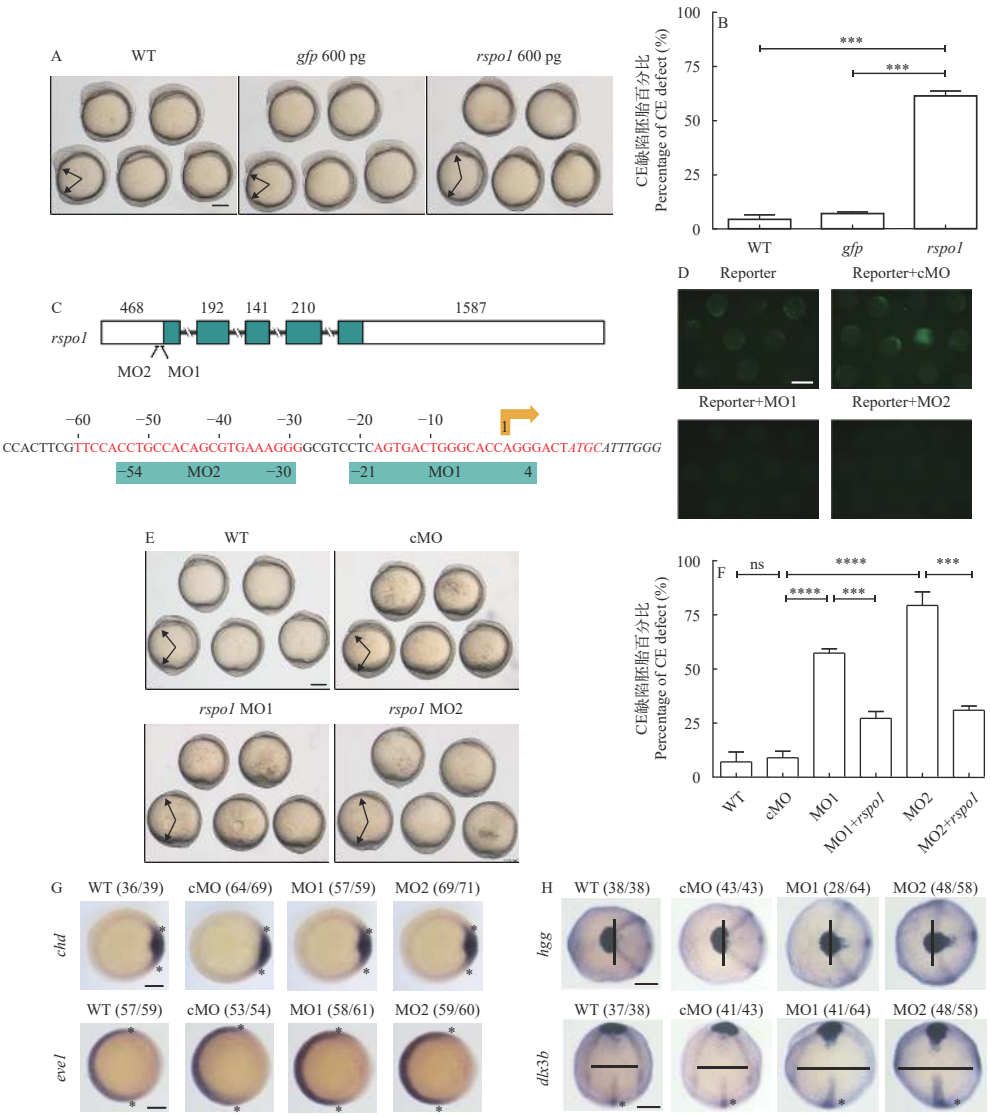

图 2　*rspo1*的过表达或敲降会影响胚胎的CE运动

Fig. 2　Overexpression or knockdown of *rspo1* impairs convergence and extension movements during gastrulation

A, B. 过表达*rspo1*导致斑马鱼胚胎CE缺陷。图A显示每组代表性的胚胎图像, 图B显示每组胚胎CE缺陷的百分率, ***表示P<0.001; C—F. 敲降*rspo1*基因导致斑马鱼胚胎CE缺陷。图C为*rspo1* MOs的结构示意图和序列, 图D为*rspo1* MOs封闭翻译的效率检测, 图E为*rspo1*敲降后12hpf胚胎表型, 图F为每组胚胎CE缺陷百分率, ns表示无显著性差异, ***表示P<0.001, ****表示P<0.0001。图示为侧面观, 背部朝右, 前端朝上, 箭头指示胚胎前端与后端的夹角。标尺: 200 μm; G. *rspo1*基因的敲降对斑马鱼胚胎背腹分化没有明显影响。图示为顶部观, 动物极朝上, 背部朝右。各组胚胎表型比例标于图中。星号表示标记基因表达范围及延展长度。标尺: 200 μm; H. *rspo1*基因的敲降导致斑马鱼胚胎CE缺陷。*hgg*为顶部观, 动物极朝上, 背部朝右, 前端朝上; *dlx3b*为背部观, 前端朝上。各组胚胎表型比例标于图中。垂直黑线表示*dlx3b*在*hgg*前面的部分, 水平黑线表示*dlx3b*标记的神经板宽度, 星号表示*ntl*的表达位置。标尺: 200 μm

A, B. Overexpression of *rspo1* cause CE defects in zebrafish embryos. Representative images of embryos at 12hpf in each group are shown in A, and the percentages of embryos with CE defects in each group are shown in B. *** means P<0.001; C—F. Knockdown of *rspo1* in zebrafish embryos led to CE defects. Schematic representation and the nuclear acid sequence of *rspo1* morpholinos are shown in C. Efficiency of zebrafish *rspo1* translation-blocking morpholinos used are shown in D. Representative phenotypes caused by morpholino-mediated knockdown of *rspo1* at 12hpf shown in E, and quantitative results are shown in F for embryos with CE defects. ns means no significant difference, *** means P<0.001, **** means P<0.0001. Lateral views with anterior up and dorsal to the right. Arrows indicate the angle between the anterior- and posterior-most embryo structures. Scale bar: 200 μm; G. Knockdown of *rspo1* have little effect on dorsoventral patterning in zebrafish embryos. Expression patterns of marker genes *chd* and *eve1* are shown. Top view with anterior up and dorsal to the right. The frequency of embryos with the indicated patterns is shown on the top of each panel. The asterisks showe the edge of the marker expression domain. Scale bars: 200 μm; H. Knockdown of *rspo1* led to CE defects in zebrafish embryos. Top view with anterior up and dorsal to the right (*hgg*), and dorsal view with anterior up (*dlx3b*). The frequency of embryos with the indicated patterns is shown on the top of each panel. Vertical black line indicates the position of *hgg* anterior to *dlx3b*, horizontal black line indicates the width of the neural plate marked by *dlx3b*. Scale bars: 200 μm

胚期维持正常CE运动所必需的。

2.2 Rspo1通过抑制Wnt/PCP信号调控胚胎汇聚延伸运动

Wnt/PCP信号通路参与胚胎的CE运动, 并通过介导转录因子ATF2和*c-jun*的表达激活JNK信号[33]。Rspo1是一种分泌型蛋白, 如果它参与Wnt/PCP信号通路, 则会影响转录因子ATF2的激活。荧光素酶报告质粒AP-1能响应ATF2的激活[34, 35], 因此, 为了探究Rspo1和Wnt/PCP信号通路之间的分子相关性, 我们使用AP-1质粒对该信号通路活性进行检测。在斑马鱼胚胎1细胞期注射不同剂量的*rspo1* mRNA, 然后在9hpf时检测AP-1报告质粒的活性。与*gfp*注射组相比, 注射*rspo1* mRNA抑制了AP-1报告质粒的活性, 其抑制作用呈剂量依赖性(图 3A)。与之相反, 斑马鱼中*rspo1*的敲降以剂量依赖的方式增加了AP-1报告质粒的活性(图 3B)。这表明斑马鱼胚胎中Rspo1能抑制JNK信号活性。为了进一步检测Rspo1是否调控Wnt/PCP信号通路, 我们研究了原肠化运动中表征Wnt/PCP信号通路活性的磷酸化JNK的水平。与对照组相比, *rspo1* 敲降的胚胎中磷酸化JNK水平显著升高(图 3C)。为了进一步研究Rspo1是否通过这一途径调节CE运动, 我

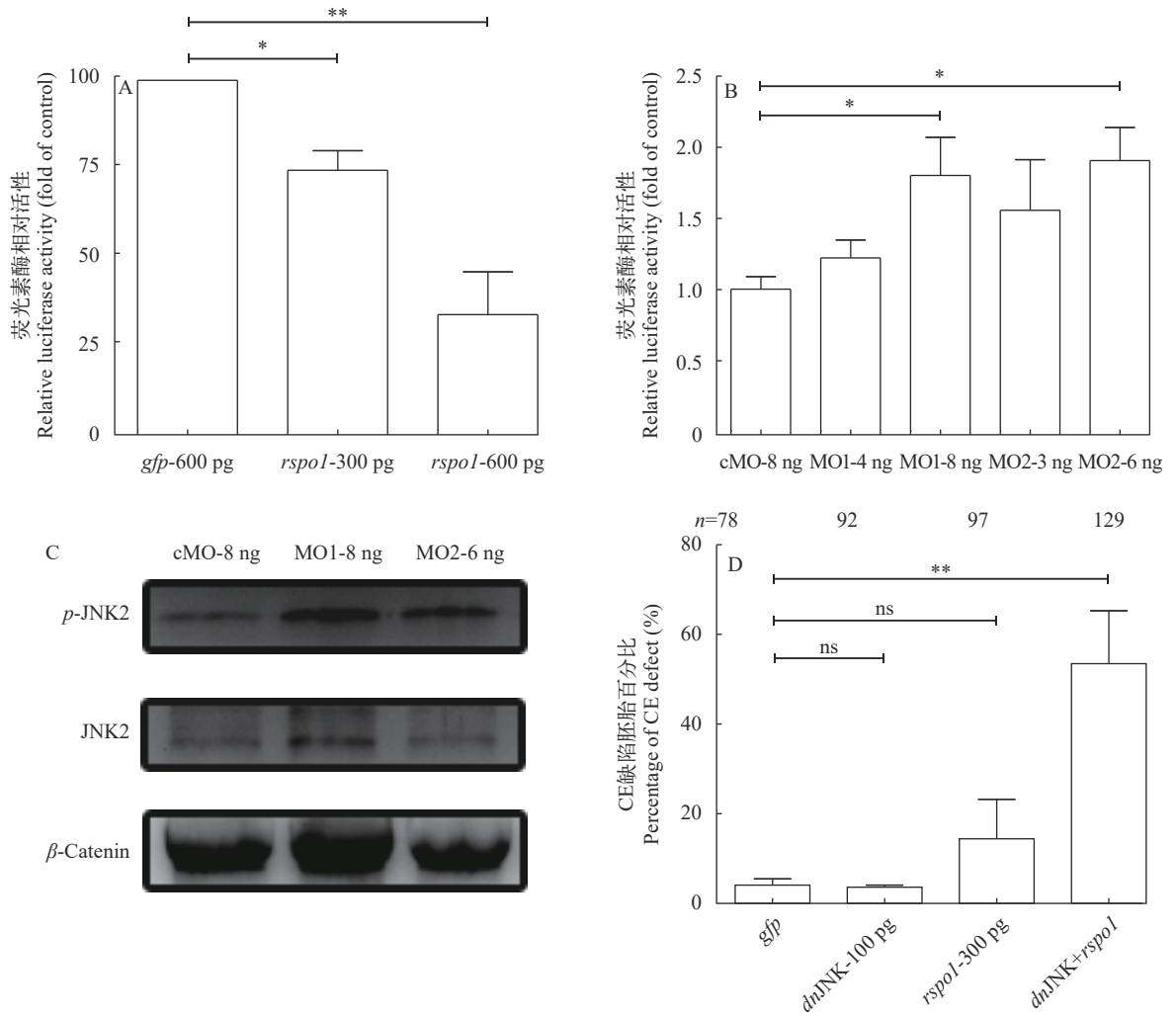

图 3 斑马鱼胚胎中Rspo1抑制Wnt/PCP信号通路

Fig. 3 Rspo1 inhibits Wnt/PCP signaling in zebrafish embryos

A. 过表达*rspo1*抑制内源Wnt/PCP信号通路活性, *表示P<0.05, **表示P<0.01; B. 敲降*rspo1*促进Wnt/PCP信号通路活性。*表示P<0.05; C. 斑马鱼胚胎中敲降*rspo1*上调JNK2的磷酸化水平。图C所示为其中一次代表性结果; D. 负显性JNK与*rspo1* mRNA协同影响Wnt/PCP信号通路。ns表示无显著性差异, **表示P<0.01。3次独立重复实验, 胚胎总数标于每组上方

A. Overexpression of *rspo1* inhibited Wnt/PCP reporter activity in a dose-dependent manner, * means P<0.05, ** means P<0.01; B. Knockdown of *rspo1* increase endogenous Wnt/PCP signaling reporter activity. * means P<0.05; C. Knockdown of *rspo1* increase the levels of phosphorylated JNK2 in zebrafish embryos. D. Dominant-negative JNK and Rspo1 have synergistic effect on Wnt/PCP signaling. ns means no significant difference, ** means P<0.01. The results are from three independent experiments, and total embryo number of each group is shown at the top of each related column

们用负显性JNK (Dominant negative JNK, dnJNK)来检测Rspo1和JNK之间的遗传互作。当单独注射低剂量的*dnJNK* mRNA (100 pg)或*rspo1* mRNA (300 pg)时, 少数胚胎出现CE缺陷(分别占总数的4%和15%; 图 3D), 而两者共注射则协同增加了CE缺陷胚胎比例(占总数的54%; 图 3D)。综上所述, 这些结果表明Rspo1通过抑制Wnt/PCP信号来调节CE运动。

2.3　*rspo1*的敲降及*wnt11*和*wnt5b*的过表达协同诱导CE运动缺陷

为了进一步证实Rspo1对Wnt/PCP信号通路的抑制作用, 我们将*rspo1* MOs与*wnt11*或*wnt5b* mRNA共同注射至斑马鱼胚胎, 观察*rspo1*基因敲降后是否能增强Wnt11或Wnt5b在CE运动中的作用。已有研究表明, 斑马鱼中Wnt11主要表达在前端轴旁中内胚层, 注射*wnt11* mRNA可导致48hpf斑马鱼眼睛呈现轻度(C1)、中度(C2)、重度(C3)的融合表型[36](图 4A)。因此, 我们通过眼睛融合程度判断*rspo1*基因敲降能否增强Wnt11在前端的作用。单独注射低剂量的*wnt11* mRNA (100 pg)只导致轻度的眼睛发育缺陷: 5%的胚胎呈现C1表型(图 4B)。单独注射低剂量*rspo1* MOs (4 ng MO1或3 ng MO2)的胚胎没有表现出此类表型(图 4B)。然而, 共注射*rspo1* MOs和*wnt11* mRNA协同影响CE运动, 显著增强了眼睛发育缺陷: 20%—25%的胚胎呈现C1表型, 20%—25%的胚胎呈现C2或C3表型(图 4B)。此外, 有研究表明*wnt5b*过量表达主要影响斑马鱼后端的汇聚延伸运动[37], 因此, 我们通过斑马鱼后端汇聚延伸运动缺陷程度判断*rspo1*基因敲降能否增强Wnt5b的作用。结果表明, 低剂量的*rspo1* MOs和*wnt5b* mRNA单独注射时不影响CE运动, 共同注射时协同产生严重的CE缺陷, 具体表现为*krox20*标记的中外侧距离变宽且*myod*标记的肌节长度缩短(图 4C—E)。因此, Rspo1和Wnt11或Wnt5b在Wnt/PCP信号通路的调控中存在遗传互作。综上所述, Rspo1在早期发育过程中能抑制Wnt/PCP信号通路。

3　讨论

在本研究中, 我们通过功能-获得和功能-缺失分析, 研究了Rspo1在斑马鱼原肠化运动中的作用。我们发现*rspo1*的过表达和敲降都会导致斑马鱼胚胎的CE缺陷。*rspo1*的过表达降低Wnt/PCP信号通路报告质粒活性, 而*rspo1*的敲降则增加该通路活性。斑马鱼胚胎中敲降*rspo1*增加内源性JNK的磷酸化水平, 这是Wnt/PCP信号激活的一个标志。相反, *rspo1*的过表达协同增强了dnJNK对原肠化CE运动的抑制作用。此外, *rspo1*的敲降可以协同增强Wnt11和Wnt5b这两个非经典的Wnt配体诱导的CE缺陷。综上所述, Rspo1通过抑制Wnt/PCP信号通路来调节原肠化运动中CE运动。

多个报道证明, Rspo1作为Lgr家族成员Lgr4/5/6的配体, 在Wnt配体存在的情况下增强Wnt/β-catenin信号水平[38—40]。值得注意的是, 一些组分在Wnt/β-catenin通路与Wnt/PCP信号通路中具有相反的功能。例如, Wnt/β-catenin通路受体LRP6的胞外区能抑制Wnt/PCP信号[41]; Wnt/β-catenin通路的抑制因子Dkk1能激活Wnt/PCP信号[42]。与Dkk1类似, Waif1/5T4、Ptk7抑制Wnt/β-catenin信号通路而促进Wnt/PCP通路[37]。我们的研究表明, Rspo1能激活Wnt/β-catenin信号通路, 但抑制Wnt/PCP通路, 其详细的分子机制还需要进一步的研究来阐明。

几项关于非洲爪蟾的体内研究表明, Rspos家族的成员Rspo3通过结合Lgrs或Sdc4 (Syndecan 4)这两种不同的受体, 既能增强Wnt/β-catenin信号, 也能增强Wnt/PCP信号[35, 40]。这与在斑马鱼胚胎中观察到的Rspo1对Wnt/PCP信号的抑制作用不一致, 推测可能的原因, 第一, Sdc4通过Rspo3介导Wnt/PCP信号通路, 而Rspo1与Sdc4之间的结合亲和力低于Rspo3与Sdc4之间的结合亲和力, 甚至未能检测到Rspo1与Syndecans之间的细胞表面结合[35]。这表明Rspo1可能不与这类受体相互作用, 因此无法促进Wnt/PCP信号通路水平。第二, Wnt/β-catenin信号的激活可能导致Wnt/PCP信号的抑制。有研究表明, 在斑马鱼胚胎中Rspo1能通过增强Wnt/β-catenin信号通路活性来特化造血干细胞[23, 24]。Rspo1在斑马鱼胚胎原肠化运动中对Wnt/PCP信号的抑制作用可能是Wnt/β-catenin信号激活导致的次级作用。第三, 斑马鱼胚胎中可能存在一种未知蛋白介导Rspo1的抑制作用, 其导致Wnt/PCP信号减弱的机制尚不清楚。

在斑马鱼中已有几项关于*rspo1*基因敲除突变体及基因敲降突变体的研究[23, 24], 但这些研究并未报道*rspo1*突变体存在CE运动缺陷。推测可能有以下几种原因。第一, 已报道的*rspo1*基因敲除突变体仅存在TSP结构域中第193位丝氨酸突变为亮氨酸, 该突变可能不足以影响Wnt/PCP信号通路[24]。第二, 母源*rspo1* mRNA可能参与发挥作用。通过TALEN及CRISPR/Cas9等技术构建的基因敲除突变体及使用的剪接阻断型MO构建的基因敲降突变体对母源mRNA影响不大。第三, 斑马鱼基因组包含*rspo1*、*rspo2*、*rspo3*、*rspo4*四个*rspo*基因, 最近

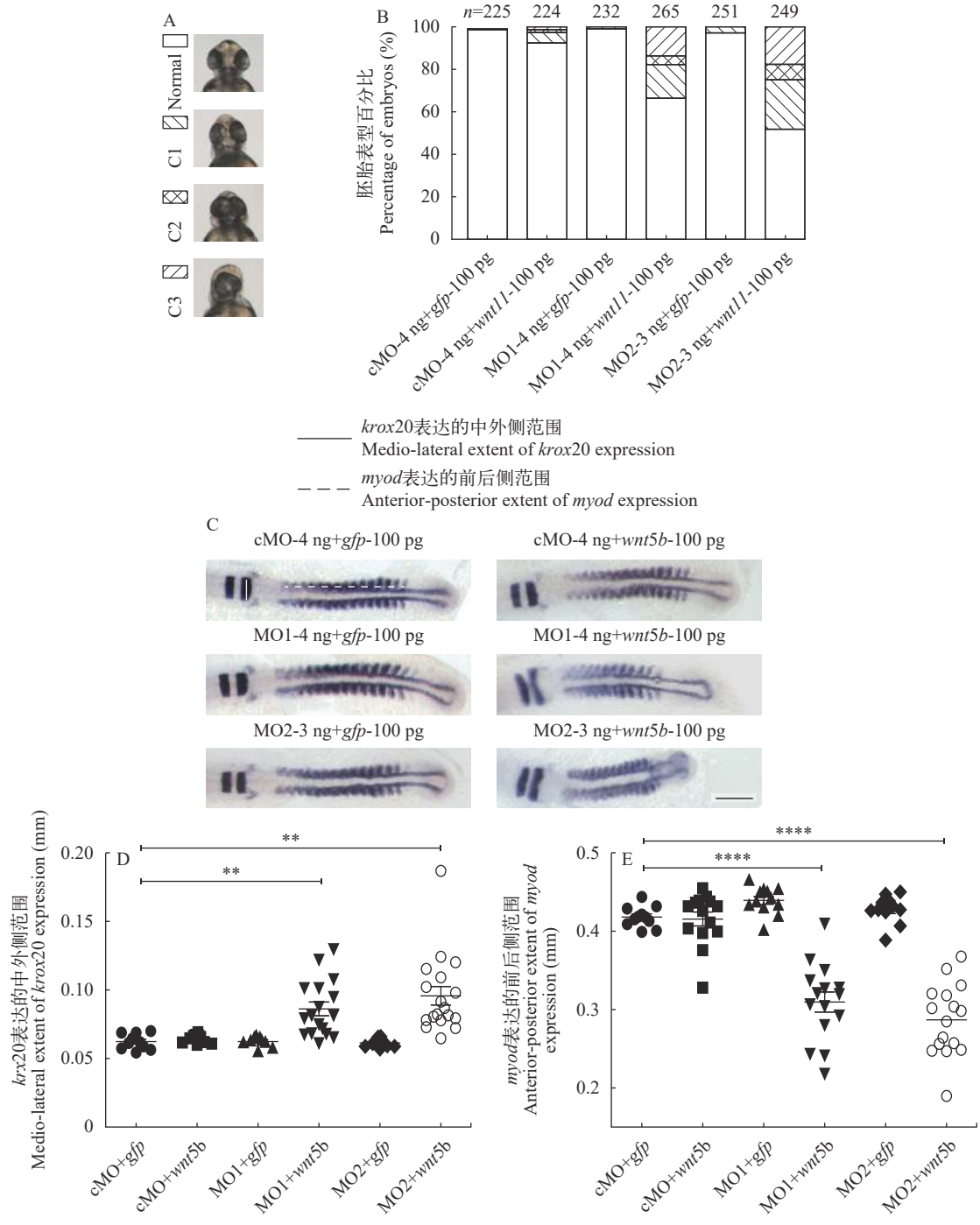

图 4 *rspo1*的敲降协同增强Wnt11和Wnt5b诱导的CE运动缺陷

Fig. 4 Knockdown of *rspo1* synergistically enhanced Wnt11 and Wnt5b induced convergence and extension defects

A. 1细胞期胚胎中显微注射cMO(4 ng), MO1(4 ng), MO2(3 ng), 或者分别与100 pg *gfp*或者*wnt11* mRNA共注射, 48hpf观察胚胎表型, 如图所示进行分类; B. 胚胎表型百分比, 表型按图A进行统计。结果来自3次独立重复实验, 统计的胚胎总数分别标示于各组上方; C. 1细胞期显微注射 cMO (4 ng), MO1 (4 ng), MO2 (3 ng), 或者分别与100 pg *gfp*或者*wnt5b* mRNA共注射, 16hpf收集胚胎进行*krox20*和*myod*标记基因的原位杂交检测。各组胚胎检测结果如图所示; D, E. 标记基因表达情况量化统计结果。*krox20*统计结果如图D所示; *myod*统计结果如图E所示, **表示P<0.01, ****表示P<0.0001。结果来自3次独立重复实验

A. 1-cell stage embryos are injected with cMO(4 ng), MO1(4 ng), and MO2(3 ng) alone, and plus 100 pg *gfp* or *wnt11* mRNA, respectively. Injected embryos are observed and categorized at 48hpf; B. the percentages of embryos shown in (A). The results are from 3 independent experiments, and total embryo numbers are given at the top; C. 1-cell stage embryos are injected with cMO (4 ng), MO1 (4 ng), and MO2 (3 ng) alone, and plus 100 pg *gfp* or *wnt5b* mRNA, respectively. Injected embryos are collected at 16hpf, and examined by *in situ* hybridization using *krox20* and *myod* probes. The representative phenotype are shown in each group; D, E. Quantitative results of marker genes expression. *krox20* (D) and *myod* (E). ** means P<0.01, **** means P<0.0001. The results are from 3 independent experiments

有研究表明, 基因敲除导致无义mRNA引起的降解可能激活同源基因的表达, 进行功能补偿, 导致突变体没有表型[43]。本研究中使用翻译阻断型MO进行*rspo1*基因敲降, 这可能也是导致表型与上述研究存在差异的原因。

综上所述, 我们的研究结果表明, Rspo1通过抑制Wnt/PCP信号通路来调节斑马鱼胚胎原肠化运动中的CE运动。Rspo1在Wnt/PCP信号通路中的作用表明, Rspos家族的功能可能比我们目前已知的更为复杂。Rspo1抑制Wnt/PCP信号转导的分子机制有待进一步研究。我们的研究为进一步探索脊椎动物中Wnt/PCP信号的调控提供了新的研究思路。

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RSPO1 IN MODULATING CONVERGENCE AND EXTENSION MOVEMENT IN ZEBRAFISH EMBRYOS

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Abstract: In vertebrate embryos, gastrulation is the fundamental morphogenetic movements. It leads to the formation of the basic germ layers and the body axis. During gastrulation, convergence and extension (CE) movements narrow a group of cells mediolaterally and lengthen them to facilitate the elongation of the anteroposterior axis. In this process, the planar cell polarity (PCP) pathway, also called the noncanonical Wnt signaling pathway, is of particular importance to control CE movements. However, the precise cellular and molecular mechanisms underlying this process remains to be further studied. Rspo1 (R-spondin 1) is a secreted protein that has been implicated in activating the Wnt/β-catenin

signaling levels with Wnt ligands, through which Rspo1 promotes angiogenesis and specifies hematopoietic stem cells, as well as promotes female development. Recently, it was reported that Rspo1 exhibits Wnt/β-catenin independent roles. For example, Rspo1-Lgr4-cAMP-Erα axis regulates estrogen receptor expression, and RSPO1-LGR5 activates TGFβ signaling in colon cancer. Given the complex role of Rspo1, we speculate that Rspo1 is involved in the early embryonic development. To investigate the developmental role of Rspo1 in zebrafish embryos, we examined the spatio-temporal expression pattern of *rspo1* using RT-PCR and whole-mount *in situ* hybridization. The results showed that *rspo1* mRNA is maternally deposited and expresses ubiquitously in early embryonic stages before 12hpf (hours post fertilization), implying that Rspo1 may play an important role in the regulation of embryonic development. Next, we carried out gain-of-function and loss-of function analysis of Rspo1. The results showed that either overexpression or knockdown of *rspo1* abrogates the CE movements during gastrulation. In order to explore whether *rspo1* affects CE movement by participating in Wnt/PCP signaling pathway, we used AP-1 luciferase reporter to monitor Wnt/PCP in zebrafish embryos. The results showed that forced expression of *rspo1* decreases but knockdown of *rspo1* increases Wnt/PCP signaling reporter activity, indicating that Rspo1 inhibits Wnt/PCP signaling pathway. Consistent with this result, the phosphorylated-JNK levels, an indicator of activity of Wnt/PCP signaling pathway, dramatically increased in *rspo1* morphant embryos at gastrulation stage. Further analyses indicate that coinjection of *rspo1* mRNA and *dnJNK* (Dominant negative JNK) mRNA, or coinjection of *rspo1* MOs and *wnt11*/*wnt5b* mRNA synergistically enhanced CE defects. Taken together, these results suggest that Rspo1 regulates CE movements during gastrulation by negatively regulating the Wnt/PCP signaling in zebrafish embryos. Overall, our studies will provide novel insights into the regulation of Wnt/PCP signaling in vertebrates.

Key words: Rspo1; Convergence and extension; Wnt/PCP signaling pathway; *Danio rerio*

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