研究简报

果蝇卵巢滤泡细胞谱系发育中的功能性microRNA筛查

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摘要 在果蝇发育过程中, microRNA(miRNA)作为负调控因子起着重要的作用。该文旨在研究microRNA在果蝇卵巢滤泡细胞谱系中的功能, 该细胞谱系因易于体内遗传操作而成为研究细胞命运决定与细胞迁移机制的良好模型。为了确认此过程中的功能性miRNA, 作者利用UAS/GAL4二元表达系统对31个果蝇miRNA进行了表型筛选。结果表明, 若干miRNA可以在卵子发生过程中引起多种严重表型。过表达与敲减miR-7均能阻断边界细胞迁移。miR-1、miR-124和miR-263b则在茎细胞诱导、边界细胞迁移或卵壳图式中发挥功能。结果表明, 该文所用基于UAS/GAL4的方法可用于确认miRNA的功能。

关键词 果蝇; 卵子滤泡细胞; microRNA; 细胞分化; 细胞迁移; 图式建成

A Screening for Functional microRNA in the Development of *Drosophila* Ovarian Follicle Cell Lineage

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Abstract As negative regulators of gene expression, microRNA (miRNA) plays important roles during *Drosophila* development. In this paper, we aimed to investigate the effects of miRNA on the commitment of *Drosophila* ovarian follicle cell lineage, which is an *in vivo* genetically tractable model to understand the mechanisms of cell fate determination and cell migration. To identify functional miRNA in the process, we performed a phenotypic screening for total 31 *Drosophila* miRNA by UAS/GAL4 binary expression system. The results showed that several miRNA cause multiple severe defects during oogenesis. And both the overexpression and knock-down of miR-7 could block the border cell migration. miR-1, miR-124 and miR-263b may function on stalk cell induction, border cell migration and egg shell patterning. The results demonstrated that UAS/GAL4 system-based strategy used in this paper was feasible for functional miRNA identification.

Keywords *Drosophila*; ovary follicle cell; microRNA; cell differentiation; cell migration; patterning

果蝇卵子发生是研究细胞命运决定、细胞运动与细胞形变的良好系统。雌性果蝇卵巢由16~20条卵巢小体(ovariole)构成, 每条卵巢小体从前端卵原区(germarium)为起始, 排列了一连串处于不同发育时期且逐步成熟的卵室(egg chamber), 直至后端输卵管(oviduct)处形成成熟的卵[1]。期间, 每个卵室都将经历14个发育时期。

在卵原区后端, 滤泡细胞前体(precursor follicle cell)向心迁移, 包裹16细胞的生殖系胞囊(16-cell germline cyst), 完成卵室的原始装配并出芽离开卵

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原区。这类细胞来源于滤泡干细胞(follicle stem cell, FSC), 随着包裹的完成分化为极细胞前体(prepolar cell)和滤泡上皮细胞(epithelial follicle cell)。前者立即退出有丝分裂, 于第1期卵室时受生殖细胞诱导, 率先分化出定位于卵室前端的极细胞(polar cell), 作为滤泡细胞命运“组织者”的极细胞则于第1期至第2期卵室间诱导其前端茎细胞(stalk cell)的分化与成熟, 由此与前端新生卵室隔离开[2]。与此同时, 滤泡上皮细胞则形成单层上皮, 包裹16个生殖细胞[15个滋养细胞(nurse cell)与1个卵母细胞(oocyte)]。滤泡上皮细胞自2期卵室起始终保持分化状态, 同时, 于第2~6期进行有丝分裂、第7~9期进行内复制(endocycle), 由此产生的多倍体才能继续适应生殖细胞持续增大的体积并始终将其完整包裹。

上皮细胞于第8期末前均保持着柱状上皮的性质, 从第9期开始, 经一系列细胞迁移与形变而重构。卵室最前端的6~8个滤泡上皮细胞受极细胞的招募, 获得边界细胞(border cell)的命运, 并与极细胞一起从滤泡上皮中脱离, 成为“边界细胞团”(border cell cluster), 沿着前–后轴的方向在滋养细胞间隙向后端迁移, 于第10期时到达滋养细胞与卵母细胞的边界处, 并最终形成精子进入的通道[3]。作为果蝇卵子发生过程中具有主动迁移能力的一类细胞亚群, 边界细胞是体内研究细胞集团性迁移的理想模型。

随着所有滤泡上皮细胞及后端定位的卵母细胞的细胞核完成迁移, 此时的卵室已经获得前–后轴和背–腹轴极性, 覆盖卵母细胞的滤泡上皮细胞进一步分化, 随着命运的不同而分泌并形成相应的卵壳(egg shell)结构。卵壳背侧附件(dorsal appendage)作为背–前端卵壳中最明显的结构, 源自于卵室背–前端的背侧附件原基(dorsal appendage primordium)细胞。这两组细胞群对称分布于宽度约为4个同时期滤泡上皮细胞的背部中线细胞(dorsal midline cell)两侧, 经过复杂的形变和重构而形成一对立体的管状结构, 背部中线细胞则将参与卵盖(operculum)的分泌[4]。

多种信号通路与功能性调节因子决定了上述滤泡细胞的分化发育。有关microRNA(miRNA)分子在其中的调控作用已见报道[5-8]。miRNA是长度约为22个核苷酸的非编码RNA, 通过转录后调控抑制其靶基因的表达。一些miRNA的表达与发育有着密切关系[9]。我们利用可获得的果蝇miRNA遗传资源, 对这些miRNA在滤泡细胞谱系发育中的功能进行了系统筛查, 获得了有价值的实验结果。

本实验采用的果蝇品系: GAL4品系: e22c-GAL4, UAS-FLP/Cyo;Tub-GAL80^{ts}/TM3,Sb购自Bloomington果蝇库, 在前期滤泡上皮细胞中高表达, 且使用GAL80^{ts}控制表达时间, 以避免胚胎及幼虫时期其他组织的表达而产生的羽化前致死现象。GR1-GAL4在中期滤泡上皮细胞中高表达, 由Schüpbach实验室惠赠。55B-GAL4购自Bloomington果蝇库, 在后期滤泡上皮细胞中高表达。Slbo^{01310},slbo-GAL4购自Bloomington果蝇库, 在边界细胞中特异性表达。其中, slbo^{01310}为边界细胞特异性增强子报告基因slbo-lacZ。

miRNA过表达品系: w;;UAS-miR-1/TM3,Sb、w;;UAS-miR-6-1,miR-6-2,miR-6-3、w;;UAS-miR-124、w;;UAS-miR-263a、w;;UAS-miR-263b、w;;UAS-miR-7、w;;UAS-miR-14、w;;UAS-miR-375、w;;UAS-miR-274、w;;UAS-miR-252、w;;UAS-miR-31b、w;;UAS-miR-278、w;;UAS-miR-964、w;;UAS-miR-79、w;;UAS-miR-9a、w;;UAS-miR-9b、w;;UAS-miR-1000、w;;UAS-miR-927、w;;UAS-miR-33、w;;UAS-miR-9c、w;;UAS-miR-184、w;;UAS-miR-92a、w;;UAS-miR-10-3p、w;;UAS-miR-210、w;;UAS-miR-999、w;;UAS-miR-276b、w;;UAS-miR-987、w;;UAS-miR-1017、w;;UAS-miR-310、w;;UAS-miR-310c和w;;UAS-miR-let-7, 本实验以w;;UAS-LUC作为阴性对照, 以上品系均购自Bloomington果蝇库。

miR-7 sponge品系: w; UAS-miR-7-EGFP-sp#2由Vactor实验室(Harvard Medical School)惠赠, 用于“吸附”并下调内源性miR-7。

本实验采用的实验材料: anti-Fas3(1:10)、anti-Orb(1:10)、anti-Armadillo(1:10)、anti-BrC(1:10)等一抗购自Developmental Studies Hybridoma Bank (DSHB), anti-SLBO(1:1 000)一抗由Goode实验室惠赠。核染料DAPI、荧光二抗anti-mouse Alexa 488、anti-mouse Alexa 546和anti-rabbit Alexa 546等购自Molecular Probe公司。PBS购自生工生物工程(上海)股份有限公司, Triton X-100购自Bio-Rad公司, 多聚甲醛购自Sigma公司, 山羊血清购自Gibco公司。

本实验采用UAS/GAL4系统, GR1-GAL4品系或55B-GAL4品系与UAS品系于25 ℃杂交, e22c-GAL4,UAS-FLP/Cyo;Tub-Gal80^{ts}/TM3,Sb品系与UAS

品系于18 °C杂交。在F1代果蝇羽化前移除培养管中的亲代果蝇。F1代果蝇羽化后挑选相应基因型的雌性果蝇, 置于含新鲜食物的培养管中继续培养。其中, GR1-GAL4和55B-GAL4果蝇的子代培养于25 °C培养箱中, e22c-Gal4,UAS-FLP/Cyo;Tub-Gal80^{ts}/TM3,Sb果蝇子代、Slbo^{01310},slbo-GAL4与miR-7 sponge杂交子代则培养于29 °C培养箱, 2~5 d后解剖检察其表型。

本实验采用免疫荧光染色, 在含10%山羊血清的PBS中解剖获得果蝇卵巢, 移入1.5 mL离心管, 吸去上层解剖液后使用1 mL 4%多聚甲醛(pH7.4)室温固定30 min, 用含0.3% Triton X-100的PBS漂洗3次。弃去上清后使用含1.0% Triton X-100的PBS室温渗透1 h。弃去渗透液后加入含10%山羊血清、0.3% Triton X-100的封闭液, 室温孵育2 h。弃去封闭液, 在相应的一抗稀释液中4 °C孵育过夜。吸出一抗稀释液后, 用含0.3% Triton X-100的PBS洗涤3次, 每次20 min, 加入1 mL封闭液室温封闭1 h。离心吸出封闭液, 加入相应的二抗稀释液, 室温孵育2 h。加入DAPI使其最终浓度达到1:2 000, 室温孵育10 min, 离心并吸出上清, 加入含0.3% Triton X-100的PBS洗涤4次, 每次20 min。涂片并封片后于4 °C避光保存。用Nikon倒置荧光显微镜Eclipse 807观察、拍照。

本实验采用Graphpad Prism 5软件进行数据分析, 显著性检验使用Student's *t*检验。

我们应用UAS/GAL4二元表达系统时空特异性地过表达或敲减(knock-down) miRNA基因, 系统筛查滤泡细胞谱系发育相关表型。本研究对总计31个miRNA进行筛查, 按卵子发生时相, 我们将获得的实验结果归纳如下。

茎细胞的命运决定与分化

利用e22c-GAL4可以在极细胞与茎细胞分化阶段过表达miRNA。蛋白Orb表达于生殖细胞且在卵母细胞中高表达[10], 而蛋白Fas 3表达于未成熟的滤泡上皮细胞, 且在极细胞中持续表达[11]。基于这两种蛋白的免疫荧光共染色, 我们可以清晰地观察每个卵室中卵母细胞的个数和极细胞的对数, 籍此判断相邻卵室是否发生融合, 并对融合卵室的数目进行统计。与阴性对照相比(e22c>LUC), 在早期滤泡上皮细胞中过表达miR-1或miR-124后, 呈现出因茎细胞缺失而产生的卵室融合现象, 即一个卵室中有三对或三对以上的极细胞, 且有两个或两个以上的卵母细胞(表1)。因此, miR-1和miR-124可能是通过影响茎细胞分化而导致了卵室的融合。

边界细胞迁移

利用GR1-GAL4分别驱动单个miRNA于中期滤泡上皮细胞中过表达, 通过边界细胞标志蛋白SLBO[12]和细胞黏附分子Armadillo的免疫荧光共染色可以观察并统计边界细胞在10期时所处的位置。我们分别以边界细胞所迁移的正常距离的0~25%、26%~50%、51%~75%和76%~100%进行定量统计。对照组内(GR1>LUC), 超过97%的第10期卵室中的边界细胞完成了迁移, 而在过表达miR-7或miR-263b后, 均有超过58%的10期卵室内的边界细胞有不同程度的迁移延迟现象(图1A~图1C, 表2)。

我们还检测了下调miRNA表达对边界细胞迁移的影响。本实验通过边界细胞特异性表达的slbo^{01310},slbo-GAL4驱动miRNA sponge来敲减miRNA的表达[13]。相对于对照组(slbo^{01310},slbo>EGFP), 过表达miR-7 sponge以下调内源性miR-7后, 10期卵室中边界细胞产生迁移延迟的表型(图2和表3)。因此, 我们推测, miR-7和miR-263b在边界细胞迁移中发挥了调控作用。

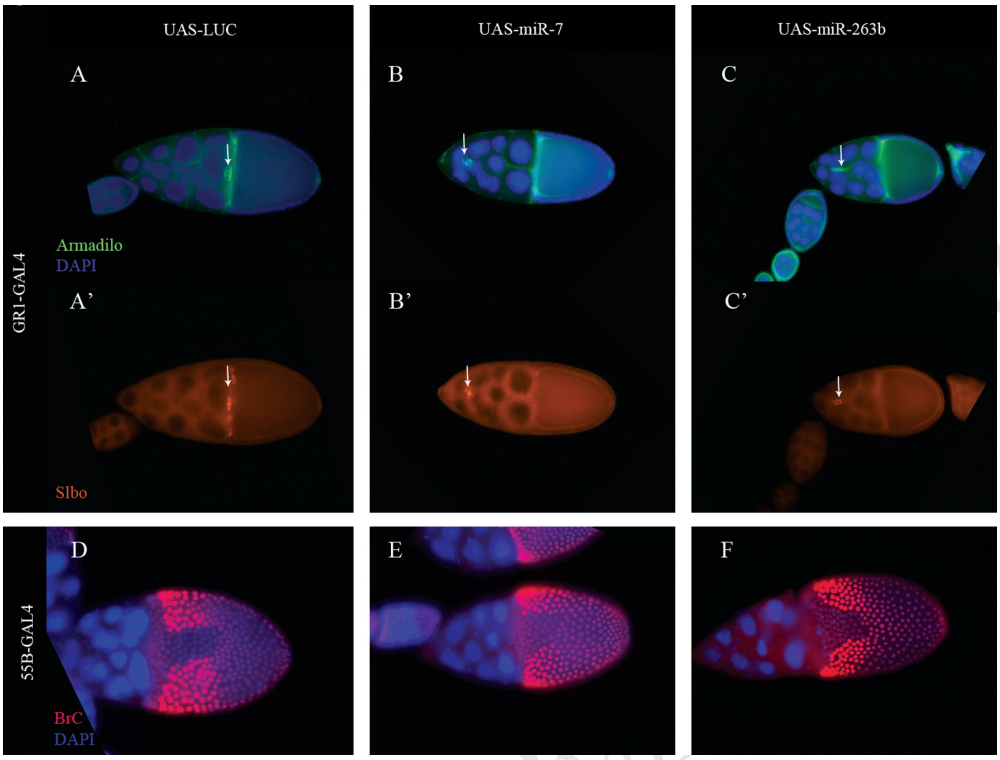
表1　过表达miR-1及miR-124的卵巢所产生融合卵室所含的卵母细胞个数及其百分比

Table 1　The number of oocytes per chamber and the frequency of fusion caused by the overexpression of miR-1 or miR-124

基因型 Genotype	每个卵室中卵母细胞的数目(%) Number of the oocyte per chamber (%)			
	2	3	4	>4
e22c>LUC (*n*=103)	0	0	0	0
e22c>miR-1 (*n*=95)	11(11.58%)**	9(9.47%)**	11(11.58%)**	17(17.89%)**
e22c>miR-124 (*n*=92)	14(15.22%)**	12(13.04%)**	9(9.78%)**	15(16.30%)**

**P<0.01, 与对照组(e22c>LUC)相比。

**P<0.01 compared with control group (e22c>LUC).



A~C': miR-7与miR-263b导致边界细胞迁移延迟, 白色箭头标识边界细胞团的位置。A~C: 细胞核(蓝色)与细胞黏附分子Armadillo(绿色)的合成图, Armadillo染色可显示卵室结构; A'~C': 以边界细胞特异性蛋白SLBO(红色)标记边界细胞团。在10期时, 阴性对照A、A'中边界细胞团已到达滋养细胞与卵母细胞的边界处, 完成迁移; B、B'与C、C'分别为过表达miR-7和miR-263b后的10期卵室, 边界细胞团均有不同程度的迁移延迟现象; D~F:为卵室背侧视图。miR-7与miR-263b导致卵壳图式建成缺陷, 图为DAPI(蓝色)与背部附件原基细胞标志蛋白BrC(红色)的合成图; 11期时阴性对照中卵室背侧中线细胞约为4个细胞宽度, 其两侧的背部附件原基细胞高表达BrC(D); E、F分别为过表达miR-7与miR-263b的11期卵室, 背侧中线细胞区域扩大。图中卵室均为前端向左。

A~C': miR-7 and miR-263b could cause border cell migration delay. Arrows indicated border cell cluster. A~C: the nucleuses were visualized with DAPI (blue). And chambers were stained with cell adhesion molecule, Armadillo (green), which could outline the structure of egg chamber; A'~C': SLBO was used to mark the border cell clusters; A,A' as negative controls, border cell cluster have reached the NC-oocyte boundary at stage 10. Stage 10 chamber with miR-7 (B,B') or miR-263b (C,C') overexpression showed border cell migration delay with varying degrees; D~F: dorsal was facing the readers; miR-7 and miR-263b could cause egg shell patterning defect. Egg chambers were stained for dorsal appendage cell marker, BrC (red) and DAPI (blue); A stage 11 chamber served as a negative control (D), in which dorsal midline cells group was about 4-cell-width and the BrC was accumulated in two groups of dorsal-anterior follicle cells flanking the dorsal midline. Stage 11 chamber with (E) miR-7 or (F) miR-263b overexpression showed enlarged dorsal midline. Anterior was to the left in all panels.

图1 过表达miR-7或miR-263b导致边界细胞迁移的延迟及卵壳图式建成的缺陷(200×)

Fig.1 Overexpression of miR-7 or miR-263b caused border cell migration delay and egg shell patterning defects (200×)

表2 过表达miR-7及miR-263b后边界细胞迁移延迟的卵室个数与百分比

Table 2 The number and the frequency of chambers with delayed border cell migration caused by the overexpression of miR-7 or miR-263b

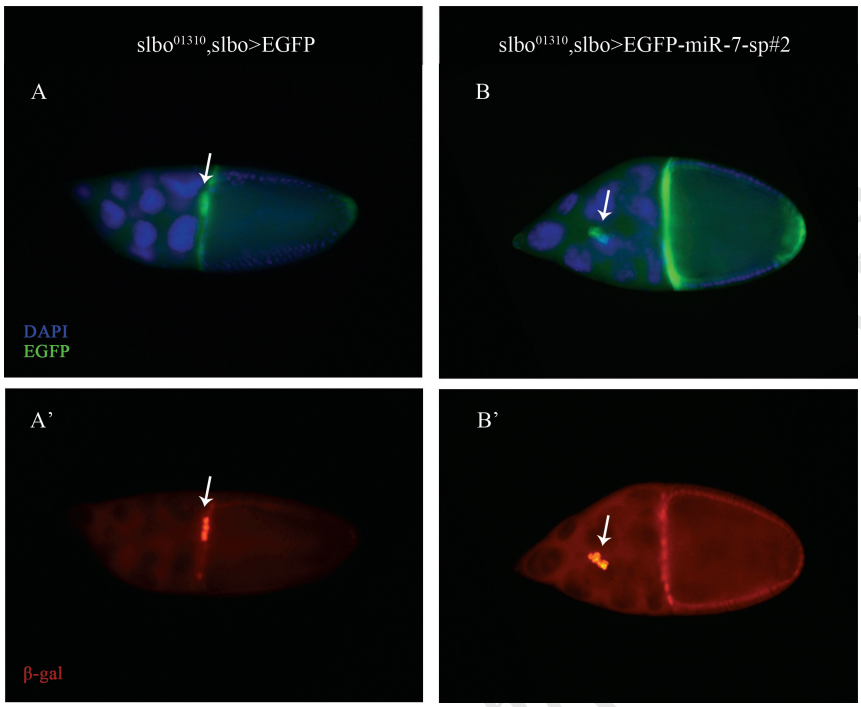
基因型 Genotype	0~25%	26%~50%	51%~75%	76%~100%
GR1>LUC (*n*=113)	1(0.88%)	0	0	2(1.77%)
GR1>miR-7 (*n*=109)	18(16.51%)**	16(14.68%)**	17(15.60%)**	15(13.76%)**
GR1>miR-263b (*n*=119)	26(21.85%)**	12(10.08%)**	12(10.08%)**	20(16.81%)**

***P*<0.01, 与对照组(GR1>LUC)相比。

***P*<0.01 compared with control group (GR1>LUC).

卵壳图式

蛋白BrC在背侧附件原基细胞中高表达, 通过对其进行免疫荧光染色, 可以观察背侧附件原基细胞的数目和分布有无异常。相比于对照组(55B>LUC), 利用55B-GAL4在后期滤泡上皮细胞中过表达miR-7或miR-263b后, 10~11期卵室背部中线



A~B': 使用边界细胞特异性的slbo^{01310},slbo-GAL4驱动下游基因表达。A、B: DAPI(蓝色)与增强型绿色荧光蛋白(绿色)的合成图; A'、B': 以边界细胞特异性增强子报告基因slbo-lacZ(红色)标记边界细胞团; 阴性对照A、A'为过表达EGFP的10期卵室, 边界细胞团完成迁移; B、B'为过表达miR-7 sponge 10期卵室中边界细胞团迁移阻滞。图中箭头指示边界细胞团所处位置且卵室均前端向左。

A~B': border cell specific line, slbo^{01310},slbo-GAL4 was used to drive the expression of downstream gene. A,B: egg chambers were stained for DAPI (blue) and EGFP (green); A',B': border cell specific enhancer trap reporter gene slbo-lacZ was shown in red and marked border cell clusters; A,A' as negative controls, border cell cluster have reached the NC-oocyte boundary on time; border cell migration was blocked with miR-7 sponge in stage 10 chamber (B,B'). Arrows indicated border cell clusters and the anterior was always to the left.

图2　miR-7 sponge介导的miR-7表达下调致边界细胞迁移延迟(200×)

Fig.2　Downregulation of miR-7 by sponge impeded border cell migration (200×)

表3　过表达miR-7 sponge后边界细胞迁移延迟的卵室个数与百分比

Table 3　The number and the frequency of chambers with delayed border cell migration caused by the overexpression of miR-7 sponge

基因型 Genotype	0~25%	26%~50%	51%~75%	76%~100%
slbo^{01310},slbo>EGFP (*n*=87)	1(1.15%)	1(1.15%)	0	3(3.44%)
slbo^{01310},slbo>miR-7-SP (*n*=83)	4(4.82%)**	7(8.44%)**	5(6.02%)**	4(4.82%)**

***P*<0.01, 与对照组(slbo^{01310},slbo>EGFP)相比。

***P*<0.01 compared with control group (slbo^{01310},slbo>EGFP).

表4　过表达miR-7及miR-263b后背部中线细胞区域扩大的卵室个数与百分比

Table 4　Frequency of enlarged dorsal midline egg chambers caused by the overexpression of miR-7 or miR-263b

基因型 Genotype	背侧中线扩大的卵室的数目(%) Number of chambers with enlarged dorsal midline (%)
55B>LUC (*n*=47)	0
55B>miR-7 (*n*=44)	15(34.09%)**
55B>miR-263b (*n*=43)	19(44.19%)**

***P*<0.01, 与对照组(55B>LUC)相比。

***P*<0.01 compared with control group (55B>LUC).

细胞数目有增多的现象, 与对照组相比增加了约3个细胞宽度(图1D和图1E、表4), 而背侧附件原基细胞数目并无异常。同时, 所产生的卵可见背部附件中间区域有扩大的现象(数据未展示)。以上结果提示: miR-7和miR-263b参与卵室背侧中线细胞的命运决定, miRNA过表达可导致卵壳图式建成的缺陷。

本实验运用UAS/GAL4表达系统对31个miRNA分子在果蝇卵巢滤泡细胞谱系发育中的功能进行了系统筛查, 发现过表达miR-1或miR-124可致相邻卵室间发生融合(可能影响卵子发生早期茎细胞的分化); 而miR-7或miR-263b的过表达不仅诱发卵子发生中期边界细胞迁移的延迟, 还导致后期卵壳图式建成的异常。我们在研究miR-7的功能时, 还从下调内源性miRNA表达的层面进行了相应的表型检测, 获得了阳性实验结果, 进而佐证了实验结果的可信性。边界细胞迁移是一个受多种信号通路调控、多步骤的复杂生物学过程。miR-7的上调或下调可能参与了对于不同信号通路的调控, 因此均得到了细胞迁移延迟的表型。基于以上研究结果, 下一步可将本文的研究策略应用到更大范围的筛查中, 以鉴定出更多参与调控滤泡细胞谱系发育的功能性miRNA分子。已知诸多信号通路如Notch[14]、JAK/STAT[15-16]、PVF/PVR[17-18]、EGFR[4,19]及BMP[4,20]等在滤泡细胞谱系发育特定过程中起关键的调控作用, 进一步的研究将探讨本文所获得的功能性miRNA分子通过调节上述信号通路组分活性而发挥功能的作用机理。

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