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Regulation of Gene Expression by the microRNA miR-124 in the Developing Nervous System of *C.intestinalis*

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Regulation of Gene Expression by the microRNA miR-124 in the Developing Nervous System of *C.intestinalis*

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Abstract: An understanding of the mechanisms by which pluripotent epidermal precursor cells differentiate and develop into mature neurons requires detailed knowledge of the gene regulatory network (GRN) underlying neuronal differentiation. Using the ascidian sea squirt *Ciona intestinalis* as a model organism, we are beginning to unravel some of the fundamental components and sub-networks of the GRN driving neuronal specification in the developing embryo. Here in particular, we have discovered putative direct gene targets of the microRNA miR-124, an important conserved transcriptional regulator specifically expressed in the nervous system, which in mammals has been shown to drive neuronal differentiation by silencing unwanted transcripts as neural progenitors develop into mature neurons. In other organisms, microRNAs regulate their gene targets by binding in a sequence-specific manner to the 3′ untranslated region (3′UTR) of their corresponding transcripts and preventing expression through RNA silencing mechanisms. Our pilot computational studies predict 22 perfect miR-124 targets and at least 298 imperfect targets. Using an *in vivo* fluorescent reporter assay, we have verified downregulation of at least one target upon miR-124 misexpression, suggesting that the mechanism of microRNA (specifically, miR-124) target downregulation via 3′UTR binding is also present in ascidians. Thus, we hypothesize that miR-124-mediated gene regulation is a fundamental mechanism governing neuronal specification in both invertebrates and vertebrates.

Keywords: microRNA, miR-124, neuronal differentiation, *Ciona intestinalis*

1 Introduction

Function and role of microRNAs
microRNAs (miRNAs) are fundamental biological molecules that have been shown to play fundamental roles in development [1], immune response [2] and cancer pathogenesis [3]. They are short (∼21 bp) non-coding RNAs that control post-transcriptional gene regulation by binding to their corresponding mRNA transcripts and preventing translation through RISC mechanisms. Growing evidence strongly suggests that miRNAs block translation specifically by binding of the first eight nucleotides, called the miRNA seed sequence, to a complementary sequence in the 3′UTR of mRNA [4]. The level of translational repression is believed to be dependent on the degree of seed complementarity, the number of seed sites, and perhaps other criteria as well [5].

Ascidian nervous system and the role of miR-124 in neuronal specification
The ascidian tadpole contains a simple version of both a central (CNS) and peripheral (PNS) nervous system. The CNS shares many characteristics of the vertebrate CNS yet only has about 100 neurons. Although few in number and possibly fixed in composition, the neurons of the CNS are capable of governing a diverse repertoire of behaviors, including light and gravity sensing and motor response to sensory stimuli [6].

The peripheral nervous system of the ascidian embryo consists of about two-dozen ciliated sensory neurons (of unknown function) located along the dorsal and ventral midlines of the tail. These cells, called epidermal sensory neurons (ESNs), generally occur in pairs and are ectodermal derivatives [7]. In the normal embryo, the epidermis is formed from a number of founder cells

that undergo a fixed number of cell divisions to form the compliment of embryonic ectoderm. In the tail, the ESNs form from one of the midline ectodermal cells that undergoes one further cell division perpendicular to the AP axis [8]. In addition to the cells that run down the tail, there are a number of ESNs located in the anterior region of the embryo (the trunk region).

Because the ascidian has a simple barebones nervous system, we believe that studying the ascidian nervous system will give us an insight into the basic, essential components of the human nervous system. Our research will focus on the early gene regulatory network controlling neuronal differentiation and development. We are particularly interested in a miRNA known as miR-124 because of its identification in many species, including humans [4], mice [9], worms [10], and fruit flies [11], and its specific, enhanced expression in neurons [4, 9, 11]. Also, a recent study on the chick embryo has shown that the microRNA miR-124 acts as a switch causing a cell to differentiate into a neuronal cell [12]. miR-124 suppresses the activity of the REST/SCP1 transcription factor complex, which acts as a global repressor of pro-neuronal genes [12, 13]. Specifically, miR-124 binds to the 3′UTR of SCP1, stopping translation, inhibiting functionality of the transcription factor complex, and causing uninhibited expression of pro-neuronal genes, driving neuronal development [12]. A separate study previously showed that REST contains many zinc finger motifs, some which mediate direct binding to DNA and others which mediate interaction with other proteins involved in its activity as a repressor of neuronal genes [14].

2 Methods

***In silico* prediction of miR-124 targets**
The C program predicting in silico miR-124 targets takes the names of two files as input arguments: a FASTA file of 3′UTR sequences and another FASTA file containing an miRNA sequence. A third program parameter indicates the desired degree of seed sequence complementarity: bp 1-8 (perfect), bp 1-7, bp 2-8, bp 2-7 or one bp error seed matching. The program then finds the number of seed-matching sites within each 3′UTR. Those sequences with at least one seed-matching site are considered targets and are output into a FASTA file.

The predicted mature miR-124 sequence used in this report was determined through previous studies in our lab (unpublished data). The 3′UTR sequences, predicted from the JGI v1.0 gene model set, were downloaded from the Joint Genome Institute Ciona intestinalis repository (http://genome.jgi-psf.org/ciona4/ciona4.download.ftp.html).

***In vivo* fluorescent reporter assays**
Fluorescent reporter assays were used to verify in vivo repression of our predicted gene targets. Briefly, reporter constructs for miR-124 and a 3′UTR target were engineered, each driven by either a ubiquitous elongation-factor (EF) or an epidermal-specific (EpiB) promoter. An EF (or EpiB)-driven control construct was also engineered containing a non-target 3′UTR. miR-124 was tagged with yellow fluorescent protein (miR-124:YFP), the UTR target with red fluorescent protein (target:RFP), and the control construct with cyan (control:CFP). Each of the constructs was then electroporated into ascidian embryos as outlined in Zeller, et.al. [15]. After electroporation, the embryos were placed on an 18 C chill plate and then assayed for fluorescence at approximately 18 hr. post-fertilization.

***In situ* hybridization**
Digoxigenin labelled RNA probes were generated from linearized templates. All embryos were reared at 18C and a standard in situ hybridization protocol [16] was employed with the following modifications: 1) hybridization reactions (pH4.5) were conducted at 56-60C for 48-72 hours, probe concentration was 0.5-5 ng/μl; 2) anti-Digoxigenin antibody (Roche) was preabsorbed against fixed embryos for at least one hour before use; 3) Blocking reagent (Roche) was dissolved in Maleic acid buffer.

3 Results

The results of the *in silico* target predictions are shown in Figure 1. Briefly, 22 targets showed perfect bp 1-8 seed matching (1 having 2 seed sites), 91 targets matched bp 2-8 (3 having 2 seed sites), 75 targets matched bp 1-7 (6 having 2 sites),

298 targets matched bp 2-7 and 280 targets matched with one bp mismatch. We also show the number of targets with multiple seed sites, since we hypothesize that those targets with multiple seed sites are more downregulated than those with only a single site. The sets of targets are not mutually exclusive; so, for example, the 22 perfect targets are also included in the bp 1-7, bp 2-8 and bp 2-7 target lists. Future studies will ensure mutual exclusivity of the target sets.

Next, we verified the predicted in silico targets using an in vivo fluorescent reporter assay. We began with the best predicted target, ci0100152283 (RFP:ci283), to see if it was downregulated when co-expressed with miR-124. This UTR target had two perfect miR-124 seed-sites, so we expected significant downregulation by miR-124, which would be indicated by a significant decrease in overall RFP target fluorescence relative to CFP control. We performed the experiment four times; twice with the constructs driven by a ubiquitous EF promoter, and twice driven by an epidermal EpiB promoter. Figure 2 shows the results of one experiment. A comparison of red fluorescence (relative to cyan control) for each embryo analyzed seems to suggest that our hypothesis is correct; namely, that miR-124 is indeed downregulating target expression via binding to seed sites within the 3′UTR. Table 2 shows the cumulative results of our experiments. The ratio of red to cyan fluorescence is significantly lower in the target embryos, indicating that miR-124 is indeed inhibiting translation of the target ci283:RFP construct. Results of statistical tests on the data are shown in the caption of Figure 3. A students t-test, permutation t-test and F-test performed on the entire data set all revealed statistical significance at the 1% level. A few other targets have been tested from the other target groups (bp 2-8, bp 1-7, one bp error). Initial results seem to indicate that the degree of downregulation is directly dependent upon the quality of binding, although more experiments need to be done for statistical significance (data not shown).

Finally, we wished to verify that miR-124 was indeed expressed in the nervous system of ascidians. We developed a digoxigenin-labelled miR-124 RNA probe which, upon in situ hybridization, binds to mature miR-124. Visualization is achieved by staining with an anti-Digoxigenin antibody. Figure 4 shows the results of miR-124 in situ hybridization. The expression pattern shown shows that miR-124 is expressed throughout the central and peripheral nervous system.

4 Conclusion

In these pilot studies we have shown the following: (1) in the ascidian, miR-124 contains about 300 predicted targets; (2) miR-124 appears to downregulate its targets via 3′UTR binding; and (3) miR-124 is expressed exclusively throughout the nervous system. It remains to be seen whether miRNA downregulation is directly dependent upon the type of seed binding and number of seed sites, although initial unpublished results seem to show a direct trend. Also, with the recent release of a new gene model set for C.intestinalis, the in silico target set will need to be updated. In vivo fluorescent reporter assays appear to be a promising method for verifying individual target repression, although a more high-throughput approach would aid in verifying miRNA targets on a global scale.

5 Figures

Degree of seed matching	Number of targets	Number with multiple sites
bp 1 - 8	22	1
bp 2 - 8	91	3
bp 1 - 7	75	6
bp 2 - 7	298	37
one bp mismatch	280	36

Figure 1: **In silico miR-124 targets**. The degree of seed matching (column 1) refers to the nucleotides in miR-124 that base pair to a target 3′UTR. The next two columns indicate the number of targets for each type of seed match, and the number of targets with more than one 3′UTR seed-matching site, respectively.

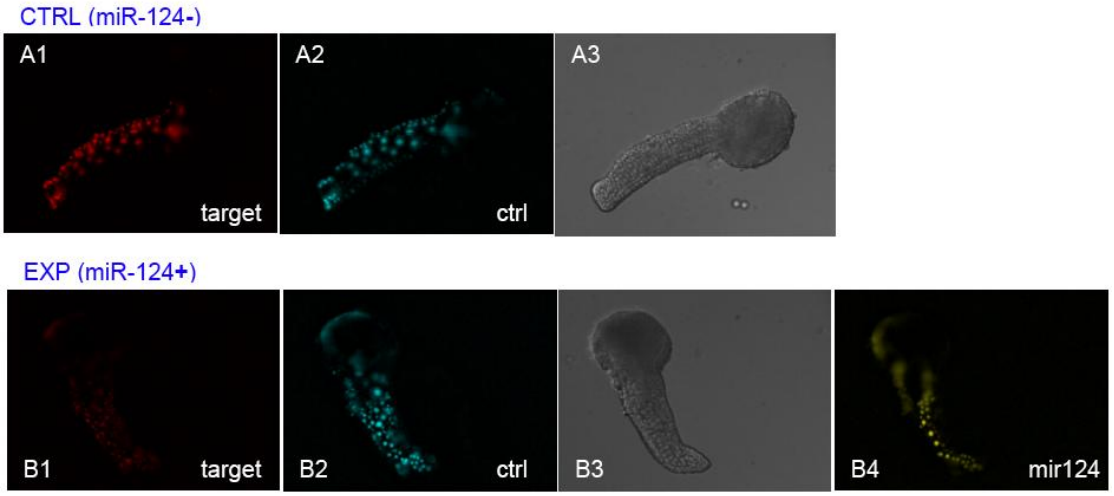

Figure 2: **Target translational repression as a result of miR-124 activity.** Upon miR-124 mis-expression, we see a decrease in red fluorescence relative to cyan control, indicating that miR-124 has blocked its translation. Images were taken between 10 and 11 h post-fertilization, at a 350 ms exposure for the control embryos and 150 ms exposure for the experimental embryos, for red and cyan fluorescence. In the experimental embryo, yellow fluorescence was captured at 2 s exposure. Mis-expressed fluorescent transgenes were driven by a ubiquitous elongation factor (EF) promoter. (A) control embryos without miR-124 mis-expression (miR-124-): (A1) RFP:ci283, (A2) CFP:ctrl, (A3) DIC; (B) experimental embryos with miR-124 mis-expression (miR-124+): (B1) RFP:ci283, (B2) CFP:ctrl, (B3) DIC, (B4) miR124:YFP.

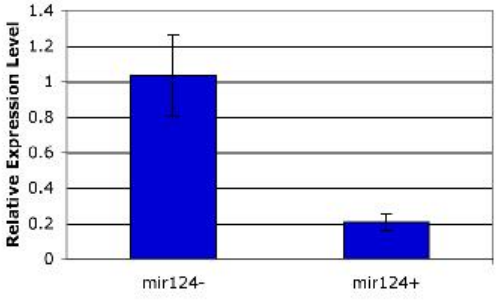

Figure 3: **Effect of miR-124 misexpression on target UTR expression level.** miR-124 was ectopically expressed in the embryo, and the ratio of target UTR (RFP) expression and control UTR (CFP) expression was calculated as the expression level for 30 embryos each in the control (miR124-) and experimental (miR124+) group. The experiment was performed a total of four times (n=4). A students t-test was performed on compiled data from all four experiments, and a p-value of 7.51x10-13 was obtained. An F-test performed on the same data set gave an F-value of 86.364, significantly above the critical value for a 0.01 significance level (F-critical = 2.299). A permutation t-test with 10,000 permutations also revealed supporting results (data not shown).

Figure 4: **In situ expression of miR-124 in neuronal cells.** Embryos fixed at 13 hrs. were treated with a probe for miR-124 mRNA and stained with NBT/BCIP. The staining pattern shows that miR-124 is expressed in neuronal cells. The punctate regions along the dorsal and ventral regions of the tail are epidermal sensory neurons, comprising the peripheral nervous system, while the large region within the head is the central nervous system.

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