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Developmentally regulated expression of Sox9 and microRNAs 124,128 and 23 in neuroepithelial stem cells in the developing spinal cord

Barbara C. Farrell*, Emmet M. Power, Kieran W. Mc Dermott

Department of Anatomy, Biosciences Institute, University College Cork, Ireland

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ABSTRACT

Central nervous system development is a complex process involving many interacting factors. MicroRNAs have recently been identified as playing key intrinsic roles in development however few of their specific targets have been identified in vivo. The transcription factor Sox9 has recently been identified as a target of miR-124 in the adult mouse sub-ventricular zone. Here we investigate the expression of the microRNAs miR-124, miR-128 and miR-23 and that of transcription factor Sox9, in neuroepithelial stem cells in the developing spinal cord. Furthermore we investigate if neurogenesis in embryonic neuroepithelial cells in the spinal cord might also be regulated by the interaction of Sox9 and miR-124. We provide evidence of the spatial and temporal regulation of miR-124, miR-128, miR-23 and Sox9, and taken together with recent findings we provide evidence that Sox9 may also be target of miR-124 in developing spinal cord neuroepithelial cells.

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1. Introduction

Since the discovery of the first microRNA (miRNA), lin4, by Lee et al. (1993) hundreds of miRNAs have been identified and knowledge of their biogenesis, functions and targets has grown immensely. MicroRNAs are small, regulatory RNA molecules 19–24 nucleotides in length (Bartel, 2004) which are proposed to be post-transcriptional regulators of gene expression and have been implicated in a number of biological processes, including development. It was originally thought that the extent of base-pairing of the miRNA to the target mRNA determined its fate (Pillai, 2005). However recent discoveries suggest the mechanism of action of miRNAs is a more complex process. It is now thought that miRNAs mediate gene regulation through multiple mechanisms and three main pathways are implicated. miRNAs may repress translationally competent ribosomes after initiation by either promoting ribosome drop-off during elongation, or by co-translational degradation of the nascent polypeptide. miRNAs may also block translational initiation through competition for the cap structure between the microribonucleoprotein (miRNP) complex and the translation initiation factor EIF4E. The final mechanism is that miRNAs promote deadenylation and degradation of their targets (for review see Liu, 2008).

Many miRNAs have a spatially and/or temporally restricted expression pattern (Kapsimali et al., 2007). They are not only expressed in a tissue specific manner but can also be restricted to individual cell types within specific tissue types (Hobert, 2004). MicroRNA tissue specificity suggests that they may play a role in the differentiation and specification of tissue specific cells (Maatouk and Harfe, 2006). The majority of human miRNAs are negative regulators of gene expression (Lewis et al., 2003). Transcription factors, proteins involved in apoptosis, and cell cycle regulators all represent possible targets (Chen and Meister, 2005). However, although many miRNA targets have been predicted using computational methods, many targets remain to be identified and verified experimentally (Kuhn et al., 2008).

A number of important central nervous system (CNS) cell specific miRNAs have been identified including miR-124, reportedly neuronally restricted, miR-128, and miR-23 which is reported to be astrocyte specific (Smirnova et al., 2005). miR-124 is the most abundant miRNA in the adult mammalian brain (Lagos-Quintana et al., 2002) and is expressed in neuronal progenitor cells and continues to be expressed into adulthood (Makeyev et al., 2007). Together with miR-9, miR-124 has been identified as a mediator of neurogenesis by stimulating neuronal differentiation of embryonic stem cells in vitro (Krichevsky et al., 2006). Hela cells transfected with miR-124 displayed altered gene expression suggesting differentiation toward a neuronal phenotype (Lim et al., 2005), specifically through

Abbreviations: miRNA, microRNA; SVZ, sub-ventricular zone; CNS, central nervous system; REST, repressor element 1 silencing transcription factor; RE1, repressor element 1; SCP1, small C-terminal domain phosphatase 1; PTBP1, polypyrimidine tract binding protein 1; E, embryonic day; Shh, Sonic Hedgehog; BMP, Bone Morphogenic Proteins.

* Corresponding author. Tel.: +353 214902246/214902247; fax: +353 214273518.
E-mail addresses: farrellbabs@gmail.com (B.C. Farrell), kmcd@ucc.ie (K.W.M. Dermott).

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down-regulation of a number of non-neuronal genes. Conversely, inhibition of miR-124 in neuronal cells resulted in an up-regulation of non-neuronal genes (Conaco et al., 2006). The first confirmed mRNA target of miR-124 was repressor element 1 silencing transcription factor (REST), which inhibits the expression of neuronal genes in non-neuronal cells (Conaco et al., 2006). Other known mRNA targets of miR-124 include the anti-neuronal factor, small C-terminal domain phosphatase 1 (SCP1) (Visvanathan et al., 2007), and the RNA binding protein, polypyrimidine tract binding protein 1 (PTBP1) (Makeyev et al., 2007). Two further identified targets of miR-124 are LAMC1, a heterotrimeric molecule formed by laminin α1, β1, and γ1 subunits, and ITGB1, which encodes an essential subunit of laminin receptors (Cao et al., 2007). However, in vivo neither inhibition nor ectopic expression of miR-124 significantly altered neuronal differentiation in the embryonic chick spinal cord (Cao et al., 2007) suggesting that miR-124 alone is not responsible for neuronal cell fate.

It has been suggested that miR-23 is astrocyte specific (Smirnova et al., 2005) however very few miR-23 targets have been demonstrated in vivo. Bioinformatics studies have shown that Hes1 is a possible target of miR-23 (microrna.org). Hes1 has been shown to inhibit neural differentiation and maintain stem cell populations by repressing proneural gene expression. Over expression of miR-23 in P19 embryonal carcinoma cells showed a decrease in Hes1 protein but not Hes1 mRNA levels (Kimura et al., 2004). Reduction in the levels of miR-23 in human NT2 cells also resulted in an accumulation of Hes1 in differentiated cells and a disruption in retinoic acid induced differentiation of NT2 cells (Kawasaki and Taira, 2003). miR-23 has also been linked to oligodendrocyte development and myelination through the regulation of lamin B1(LMNB1) (Lin and Fu, 2009).

Sox9 is a member of the E group of Sox proteins along with Sox8 and Sox10 and is strongly expressed, initially in neural stem cells and later on in glial cells of the developing CNS. Sox9 is essential for the proper development of both oligodendrocytes and astrocytes, but not neurons, and has also been implicated in the mechanism that causes neural stem cells to switch from neurogenesis to gliogenesis (Stolt et al., 2003). It has also been reported that Sox9 is present in a small population of subventricular zone (SVZ) cells in the postnatal and adult mouse brain (Kordes et al., 2005). It has recently been shown that miR-124 is an important regulator of the temporal progression of adult neurogenesis in the mouse subventricular zone and that Sox9 is a target of miR-124 at the transition from transit amplifying cell to neuroblast stage in the adult mouse brain (Cheng et al., 2009).

Here in order to further understand the developmental regulation of cell fate we compare the temporal expression of miR-124, miR-128 and miR-23 in the developing spinal cord. The temporal, spatial and cellular expression of miR-124 and Sox9 is further compared to evaluate if, in spinal cord neuroepithelial stem cells, Sox9 maybe a potential target for regulation by miR-124. Such regulation would suggest that a potential mechanism governing neurogenesis in the developing spinal cord is the inhibition of Sox9 by miR-124 in mostly gliogenic neuroepithelial progenitor population.

2. Experimental procedures

For immunohistochemistry and in situ hybridization, Sprague-Dawley rat embryos, aged from embryonic day 10 (E10) to E16 were immerse fixed in 4% paraformaldehyde, cyroprotected in 30% sucrose, embedded in optimal cutting temperature compound (OCT), and frozen in liquid nitrogen chilled iso-pentane. Tissue was stored at −80 °C prior to cryostat sectioning at 15 μm.

For RNA isolation, Sprague-Dawley rat spinal cords were removed via dissection from embryos and pups, aged E10 to postnatal day 2 (P2), and stored at −80 °C prior to RNA isolation. All work was approved by the ethical approval board at University College Cork.

2.1. RT-PCR

RNA was isolated, from E10 to P2, tissue using mirVANA RNA isolation kit (Ambion) according to the manufacturer's instructions. Subsequently end point RT-PCR was carried out using mirVANA RT-PCR kit (Ambion) according to the manufacturers instructions and using primers for miR-23, miR-124 and miR-128 (Ambion). All the RT-PCRs were repeated three times with three different sets of samples.

2.2. In situ hybridization

In situ hybridization was carried out using DIG-labelled cDNA probe for Sox9 prepared from Images clone 3666566. The miR-124 and miR-23 probes were also DIG labelled (Exiqon). In situ hybridization was carried out over 3 days on sections obtained from E10, E11, E12, E14 and E16 rat spinal cord.

Slides containing sections were removed from the −80 °C freezer and the sections were thawed for approximately 1 h at room temperature. Pre-hybridization and hybridization solutions were warmed to 65 °C. Slides were placed into slide-mailers (Cell Path) and washed once with PBT for 5 min. Sections were fixed using 4% PFA in PBT for 20 min at room temperature then washed with PBT (2 × 5 min). Pre-hybridization solution was added for 1 h at 65 °C. The in situ probe for Sox9, miR-124 or miR-23 was diluted in hybridization solution and denatured for 5 min at 85 °C. 100 μl of Sox9 probe or miR-124 probe or miR-23 probe was added to each slide and hybridized overnight at 65 °C.

Wash solution 1 and wash solution 3 were heated to 65 °C for 30 min. Slides were washed with solution 1 (3 × 15 min), and then with solution 3 (3 × 15 min). Slides were washed with TBST (3 × 5 min) and then incubated in 10% sheep serum (Sigma) in TBST for 60 min at room temperature. 100 μl of anti-DIG antibody (Roche) (1:2000 in 1% sheep serum in TBST) was added to each slide. Sections were incubated overnight at 4 °C.

Sections were washed with TBST (3 × 5 min) and subsequently (4 × 15 min). Fresh NTMT was made and used to wash the slides (3 × 10 min). Sections were incubated in NBT/BCIP solution (Roche) in the dark and the reaction was monitored. When the reaction was completed the slides were washed with NTMT (2 × 10 min), and then with PBT, pH5.5, for 10 min. Slides were washed with PBS (2 × 10 min). Sections were post-fixed with 4%PFA in PBS for 30 min and washed with PBS (2 × 10 min). Sections were left to dry at room temperature. Slides were then mounted in DPX mounting medium (Fluxa). Sections were imaged using Kohler illumination bright-field microscopy on an AX70 microscope (Olympus).

2.3. Immunohistochemistry

Sections were washed with PBS (3 × 3 min) then blocked with 20% normal goat serum in PBS (10 mM) with 0.02% Triton-X-100 for 30 min. Sections were incubated with the primary antibody (Sox9, a gift from Dr. M. Wegner) in PBS containing 1% NGS and 0.02% Triton-X-100. Sections were covered with a maximum of 50 μl of solution and placed at 4 °C overnight. Sections were washed with PBS (3 × 3 min), then incubated with the appropriate secondary antibody, either Alexa Fluor 488 donkey anti-rabbit IgG. Secondary antibodies were diluted in PBS with 1% serum & 0.02% Triton-X-100 and left for 1 h at room temperature in darkness. Sections were washed with PBS (3 × 3 min). The nuclear dye bisbenzimide at a concentration of 1:2000 was added to the sections and they were placed at room temperature in the dark for 4 min before rinsing again with PBS. Slides were then cover-slipped and imaged.

3. Results

3.1. The expression of miR-23, miR-124, and miR-128 in developing spinal cord tissue

Using reverse transcriptase (RT)-PCR we examined the expression of the miRNA miR-23, and the neuron specific miRNAs miR-124 and miR-128 at intervals from embryonic day (E) 10 to postnatal day (P) 2. All three miRNAs are expressed in the developing spinal cord and each is differentially expressed (Fig. 1). It has been reported that miR-124 is the most abundant miRNA in the mammalian brain (Lagos-Quintana et al., 2002) and of the three miRNAs examined here miR-124, along with miR-23, was expressed earliest in developing spinal cord tissue.

Based on indications in the literature that miR-124 is neuronally specific and miR-23 is possibly expressed in astrocytes then weak expression at E10 suggests that some neuroepithelial progenitor (NEP) cells at this age may already be lineally committed. This raises the interesting question as to whether miR-124 and miR-23 expressing neuroepithelial cells are already predetermined to be

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