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MiR-3099 is Overexpressed in Differentiating 46c Mouse Embryonic Stem Cells upon Neural Induction

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Abstract

Background: **MicroRNAs (miRNAs) have a crucial role in gene expression regulation and protein synthesis, especially in the central nervous system. In developing mouse embryos a novel miRNA, *miR-3099*, is highly expressed, particularly in the central nervous system. This study aims to determine the expression of *miR-3099* during cellular differentiation of 46C mouse embryonic stem cells after neural induction with N2/B27 medium.**

Methods: **46C mouse embryonic stem cells were subjected to neural induction with N2/B27 medium. At 0, 3, 7, 11, 17, and 22 days after neural induction, the cells were screened for various pluripotent, progenitor, and differentiating/differentiated cells markers by immunocytochemistry and reverse-transcriptase polymerase chain reaction (RT-PCR). Stem-loop pulse RT-PCR was performed to determine the expression of *miR-3099* at all selected time points after neural induction.**

Results: **Our findings showed that after induction, mouse embryonic stem cells differentiated into heterogeneous pools of cells containing neurons, astrocytes, and oligodendrocytes. Mouse embryonic stem cells and neural progenitor/precursor cells were also present in culture up to day 22 as indicated by RT-PCR analysis. Elucidation of *miR-3099* expression during in vitro neural induction revealed that this miRNA was expressed throughout the differentiation process of 46C mouse embryonic stem cells. *miR-3099* was expressed at higher levels on day 11, 17, and 22 as compared to day 0, 3 and 7 after neural induction.**

Conclusion: **The level of *miR-3099* expression was higher in differentiated mouse embryonic stem cells after neural induction. This finding suggested that *miR-3099* might play a role in regulating neural stem cell differentiation. However, further characterisation of *miR-3099* in a better characterised or optimised differentiated neural stem cell culture would provide increased understanding of the cellular function and molecular targets of *miR-3099*, especially in neuron development.**

Keywords: *MicroRNA, miR-3099, neural induction, 46C mouse embryonic stem cells, neuron*

Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNA of 18-24 nucleotides (nt) that are emerging as key regulators of many post-transcriptional processes (1,2). miRNAs serve as repressors in the translation process by inhibiting the assembly of ribosomal complexes or promoting deadenylation processes on target mRNAs (1,2). miRNAs have crucial roles in regulating the development of the nervous system (3–6). For example, *miR-124*, *let-7b* and *miR-137* play an essential role during differentiation of neurons in mice (7–11). *miR-124* is one of the most extensively studied miRNAs that is involved in regulating the function and development of the nervous system. *miR-124* promotes neuronal differentiation by regulating an intricate network of nervous system-specific alternative splicing as well as by participating in adult neurogenesis of the stem cell niche in the subventricular zone of the mouse brain (12,13).

In 2011, *miR-3099* was discovered in a deep sequencing analysis of small RNA isolated from the developing E15.5 mouse brain (14). *miR-3099* was found to be expressed in mouse embryonic stem (mES) cells, blastocysts and throughout the whole mouse embryo between E9.5 and E11.5, suggesting that this miRNA could have a broad regulatory role. At E13.5, its expression was confined to the cortical neuroepithelium, striatum, medial pallium (hippocampal allocortex), and the subventricular/ventricular zone of the superior and inferior colliculi. The expression of *miR-3099* was further restricted to the cerebral cortex cortical plate in E15.5 embryos. In whole brains from E17.5 embryos, *miR-3099* expression was observed in the cortical plate, piriform cortex and was expressed at lower levels in the hippocampal formation, where neurons are predominantly found (14). The specific expression pattern of *miR-3099* in the central nervous system during the late stages of embryonic development suggests that this miRNA may have a crucial role in regulating key markers involved in neurogenesis.

In this study, we aimed to determine the expression of *miR-3099* in an in vitro model prior to pinpointing its function in neurogenesis. We used the *Sox1*-green fluorescence protein (GFP) knock-in 46C mES cell line developed by Austin Smith (15) to evaluate *miR-3099* expression after neural induction. 46C mES cells are pluripotent and can differentiate in vitro into different types of cells, which makes them suitable for neural differentiation studies after neural induction (16). In 46C mES cells the pan-neural gene *Sox1* confers cell-autonomous green fluorescence and puromycin resistance once *Sox1* is expressed (17). The *Sox1* gene is a specific marker for mammalian neural progenitors such that GFP expression in 46C mES cells would serve as an indicator of successful induction of 46C cell differentiation into neural progenitors. This property enables the purification of both neural and non-neural cells that are generated during neural differentiation of these cells (17).

Materials and Methods

Embryonic stem cell culture

The 46C mES cells were a gift from John Mason (University of Edinburgh, United Kingdom). The cells were routinely cultured in 1X GMEM (BHK-21; Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco), 1% MEM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 2 mM L-glutamine (Gibco) and 10 µg ml-1 human recombinant leukaemia inhibitory factor (LIF; Millipore) on a 0.1% gelatin (Sigma) coated plate. The cells were sub-cultured every other day when the cells were 70-80% confluent.

Neural differentiation assay

Neural differentiation was carried out using a monolayer differentiation protocol adapted from Ying et al. (18). Undifferentiated mES cells were dissociated and cultured on a 0.1% gelatin-coated plate in DMEM/F12 supplemented with N2 (Gibco). After 24 hours, half of the medium was removed by aspiration and replaced with a 1:1 ratio of DMEM/F12-N2 and neurobasal-B27 (N2/B27; Gibco). The N2/B27 medium was changed every other day.

Immunocytochemistry

Immunocytochemistry (ICC) was conducted in 24-well plates. The attached cells were fixed in 4% paraformaldehyde (PFA; 50 mM NaOH, 1X PBS) for 30 minutes before permeabilization in 1% Triton-X100 for 15 minutes at room temperature (RT). Cells were then blocked for 30 minutes at RT (0.3% bovine serum albumin, 1% appropriate serum, 0.1% Tween-20 in 1X PBS) prior to incubating the cells in 1/200 dilution of primary antibody *Tuj1* (Sigma) at 4 °C overnight. After rinsing with 1X PBS twice, the cells were then incubated with a 1/200 dilution of Alexa Fluor 488 donkey anti-mouse IgG (H+L; Life Technologies) secondary antibody for 1–2 hours at RT in the dark. The cells were counterstained with propidium iodide (Sigma) for 10 minutes at RT after rinsing with 1X PBS twice. The cells were then left in PBS in the dark until visualisation with an IX51 inverted fluorescent microscope (Olympus).

Reverse transcriptase-polymerase chain reaction

Isolation of total RNA from the stem cells was performed using TRIzol® Reagent (Invitrogen) according to the manufacturer's protocol under RNAse free conditions. Reverse transcription of *miR-3099* was performed on 0.5-1.0 µg of total RNA using the Superscript® III Reverse Transcriptase kit (Invitrogen) according to a modified protocol as described previously (14). Briefly, a stem-loop primer (5′-GTTGGCTCTG GTAGGATGCC GCTCTCAGGG CATCCTACCA GAGCCAACTC CCCA-3′) was used to prime *miR-3099*. The reverse transcriptase-polymerase chain reaction (RT-PCR) was then performed using a specific forward primer for *miR-3099* (5′-CGCGTAGGCT AGAGAGAGGT-3′) and a universal reverse primer for the stem-loop primer (5′-GTAGGATGCC GCTCTCAGG-3′) in 1X LC480 Probe Master Mix (Roche) according to Ling et al. (14) with the pre-amplification step omitted. Reverse transcription of other genes (16 genes) was performed on oligo-d(T)20 primed reactions. RT-PCR was then carried out in 1X LC480 Probe Master Mix (Roche) according to the manufacturer’s protocol. The primers used are summarized in Table 1. PCR reactions were performed in an Eppendorf Mastercycler® gradient (Eppendorf) PCR machine with a pre-denaturation step at 94 °C for 10 minutes followed by 30–35 cycles of 94 °C for 10 seconds (denaturation), 60 °C for 30 seconds (annealing) and 72 °C for 10 seconds (elongation) with an additional elongation step at 72 °C for 5 minutes. Qualitative RT-PCR analysis was performed on an ethidium bromide stained 2% (w/v) agarose gel after electrophoresis.

Table 1: Summary of primers used for RT-PCR analysis

Gene		Primers (5’ → 3’)	GC%	Tm (°C)	Annealing (°C)	Amplicon size (nt)
Pgk1	Forward	TACCTGCTGGCTGGATGG	61	60	60	65
	Reverse	CACAGCCTCGGCATATTTCT	50	60		
Hmbs	Forward	AAAGTTCCCCAACCTGGAAT	45	59	60	98
	Reverse	CCAGGACAATGGCACTGAAT	50	60		
Oct4	Forward	CACGAGTGGAAAGCAACTCA	50	60	60	129
	Reverse	GCTTTCATGTCCTGGGACTC	55	59		
Nanog1	Forward	TGCTTACAAGGGTCTGCTACTG	50	59	60	76
	Reverse	GAGGCAGGTCTTCAGAGGAA	55	59		
Sox2	Forward	CGCCCAGTAGACTGCACA	61	59	60	95
	Reverse	CCCTCACATGTGCGACAG	61	59		
Sox1	Forward	TTGAGGCAGCTGGGTCTC	61	60	60	75
	Reverse	GCTGTTGTCCCTATCCTTGG	55	59		
Nestin	Forward	TCCCTTAGTCTGGAAGTGGCTA	50	60	60	68
	Reverse	GGTGTCTGCAAGCGAGAGTT	55	60		
Tuj1	Forward	GCGCATCAGCGTATACTACAA	48	59	60	85
	Reverse	CATGGTTCCAGGTTCCAAGT	50	59		
Mtap2	Forward	TCCTCCATCCTCCCTCCT	61	59	60	114
	Reverse	GCGAATTGGTTCTGACCTG	53	59		
Neurod1	Forward	GGGAACAGCCTTACCCTTGT	55	60	60	67
	Reverse	CCACCAGAAATCACCAGGAG	55	60		
Sox4	Forward	ACAGCGACAAGATTCCGTTC	50	60	60	62
	Reverse	GTCAGCCATGTGCTTGAGG	58	60		
Sox11	Forward	GTGGCGGTCAGGATAAAGAG	55	59	60	75
	Reverse	TCTCAGCGCCACATCTCTC	55	60		
NeuN	Forward	CTACACACCCGCACAGACTC	60	59	60	105
	Reverse	GTCTGTGCTGCTTCATCTGC	55	59		
Gfap	Forward	CGCCACCTACAGGAAATTG	53	59	60	76
	Reverse	CTGGAGGTTGGAGAAAGTCTGT	50	59		
Olig1	Forward	GCGTCCTTTCCTTGTCCAG	58	60	60	76
	Reverse	CCTCCTAGATCCGCATGGT	58	60		
Cspg4	Forward	GGCCGTGATGGTGTCTTT	56	59	60	111
	Reverse	GCAGCCACAGTGATCTTGG	58	60		

Results

In this study 46C mES cells were cultured on gelatin-coated plates for 22 days after neural induction using N2 and B27 supplements (Figure 1a). The mES cells differentiated into neural progenitor cells on day 3 (Figure 1b). On day 7, the cells differentiated into neuronal precursor cells (Figure 1c) and continued to differentiate into a mixture of cells, including those resembling immature and mature neurons on day 17 (Figure 1d) and 22 (Figure 1e), respectively.

To ascertain that the 46C cells differentiated into neurons, we performed ICC on cells 7 days after neural induction. The 46C mES cells responded to neural induction by developing and expressing markers for neural precursor and immature neuronal cells (Figure 2). Immunolabeling was performed on day 7 after induction, and revealed cells labelled for *Tuj1*, a marker for immature neurons (Figure 2a, 2b). Neural precursor cells expressing *Sox1* were also observed as GFP positive cells 7 days after neural induction (Figure 2c, 2d). The 46C transgenic mES cells have a knock-in GFP (green fluorescent protein) within the *Sox1* gene locus, which served as a reporter gene that represents the expression of *Sox1* (15). These observations suggest a successful induction of mES cells in the neural stem cell lineage.

We further examined the expression of various markers at the transcript level using 46C mES cell cultures at various time points after neural induction. Qualitative RT-PCR was performed to evaluate markers for stem cells

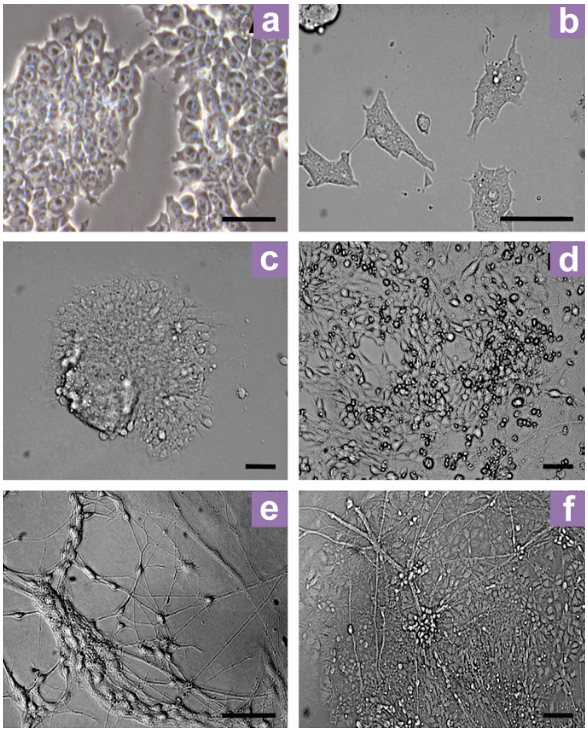

Figure 1: Mouse embryonic stem cell differentiation after neural induction at (a) day 0 (mouse embryonic stem cells), (b) day 3 (neural progenitor cells), (c) day 7 (precursor cells), (d) day 11 (immature neural cells), (e) day 17 (immature/differentiating neural cells) and (f) day 22 (differentiating/mature neural cells). The scale bar represents 50 µm for all micrographs except for (c), which represents 100 µm.

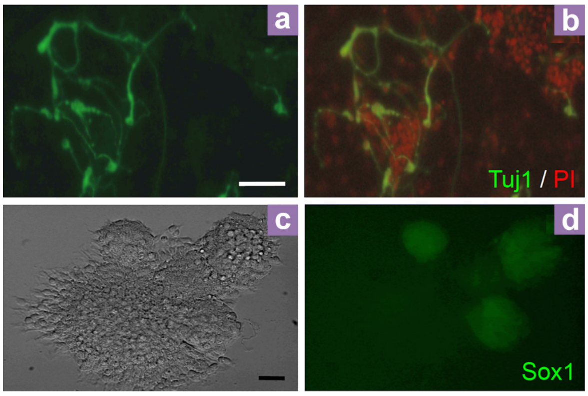

Figure 2: (a,b) Immunocytochemistry at day 7 after induction by using antibody against *Tuj1* the special marker for immature neurones. (c,d) *Sox1* expression was detected on cells 7 days after neural induction. 46C transgenic mouse embryonic stem cells bear a knock-in GFP (green fluorescent protein) that fluoresce under UV excitation, which served as a reporter gene that represent the expression of *Sox1*. PI denotes propidium iodide and the scale bar represents 50 µm for micrographs (a) and (b) whereas represents 100 µm for micrographs (c) and (d).

(*Oct4, Nanog* and *Sox2*), neural progenitor cells (*Sox1* and *Nestin*), immature neurons (*Tuj1*), differentiating neurons (*Mtap2*, *Sox4* and *Sox11*) mature neurons (*NeuN* and *NeuroD*), astrocytes (*Gfap*) and oligodendrocytes (*Olig1* and *Cspg4*; Figure 3). Our results showed that the expression of undifferentiated cell and neural progenitor cell markers was present at all the time points, whereas markers for both immature and differentiating neurons were seen from day 3 and onwards with obvious expression observed on days 11–22. Expression of mature neuronal markers was first observed on day 11 as indicated by *NeuN*, which together with *NeuroD* became significant from day 17 and onwards. A proportion of cells differentiated into astrocytes as indicated by *Gfap* expression on days 17–22. We also observed only weak expression of *Olig1* for oligodendrocytes on day 22. Qualitative RT-PCR analysis indicates a heterogeneous population of cells in 46C mES cell cultures at 3–22 days after neural induction.

Using the same RNAs isolated from 46C mES cell cultures at various time points after neural induction, qualitative stem-loop RT-PCR was performed to evaluate the level of *miR-3099* expression. This analysis showed that *miR-3099* was expressed at all-time points assessed (Figure 3), and was upregulated starting on day 7 after neural induction. The expression was approximately 2-3-fold higher on days 17 and 22 when compared to 0–3 days after neural induction.

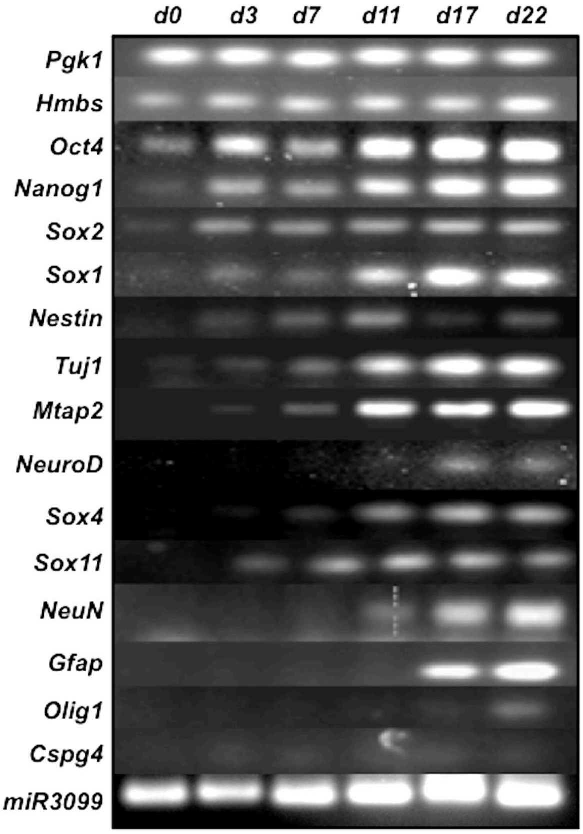

Figure 3: Semi quantitative RT-PCR cells on total RNAs purified from mES cells on day 0, 3, 7, 11, 17, and 22 after neural induction for various markers and *miR-3099* expression.

Discussion

In this study we found a heterogeneous population of cells was present in mES cell cultures 22 days after neural induction. This observation concurs with previous neural differentiation studies involving mES cells (19) and human embryonic stem cells (20). The coexistence of stem cells, precursor cells and differentiated cells such as neurons, astrocytes, and oligodendrocytes exemplifies the heterogeneity of cell lineage specification and development in the culture. As demonstrated in previous studies (19,20), pluripotent cells were found together with cells from various lineages that matured at different rates and in turn leads to cell type heterogeneity in culture. In addition, our analysis at the RNA level of various markers for different cell types such as stem cells, neural precursor cells and differentiating/differentiated cells is in agreement with previous studies (16,20–25). To date, the characterisation of mES cells using various markers has been well-established. *Oct4* and Nanog mRNA were upregulated during the pluripotent stage of mES cells (21), whereas *Sox1* and *Nestin* proteins were expressed in neural precursor cells (16,22). After neural induction, *Tuj1*, *NeuN*, *Gfap*, and Olig2 markers were expressed in differentiating neurons, mature neurons, astrocytes and oligodendrocytes, respectively (20,23–25). Our study also included additional markers for different cell types across the differentiation process, including *Sox2* (stem cells), Map2, *Sox4*, *Sox11* (differentiating neurons) and *NeuroD* (mature neurons), with expression levels that were in line with the commonly used markers described above.

miRNAs play a very important role during the modulation of ES cell maintenance and differentiation (26,27). At least a dozen miRNAs have been found to be differentially expressed as pluripotent ES cells differentiate or commit and differentiate into neural lineage cells (27). miR-9 and miR-134 were both implicated in the regulation of neural stem cell self-renewal and fate determination as well as epigenetic regulation of neural stem cell proliferation (28). In this study, the *miR-3099* expression profile supported previous transcript analyses performed on mES cells as well as neuro-differentiating P19 teratocarcinoma cells (14). Upregulation of *miR-3099* in the cell culture model containing differentiating and differentiated/mature cells of a neural lineage suggests that this miRNA plays a crucial role in neural differentiation. Concurring with *miR-3099* localisation to brain regions consisting of mainly neurons in an early embryonic development stage (14), we propose that the *miR-3099* functions as a key regulator of neurogenesis processes.

Conclusion

In this study, *miR-3099* was found to be upregulated in differentiating 46C mES cells 7 days after neural induction, indicating that this miRNA may play an essential role during neuron differentiation. However, we could not exclude the possibility that the proliferation of stem cells after neural induction may contribute to this upregulation. Therefore, a better characterised or optimised differentiated neural stem cell culture would allow a more refined analysis of the cellular function and molecular targets of *miR-3099*, especially during neuron development.

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Conflict of Interest

None.

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Authors' Contributions

Conception and design: KHL
Analysis and interpretation of the data, final approval of the article: KHL
Drafting of the article: SZA, KHL
Critical revision of the article for the important intellectual content: PSC, RR, NN, KHL
Administrative, technical or logistic support: NN, KHL
Collection and assembly of data: MA, CMN, WHS

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