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miR-744 and miR-224 Downregulate Npas4 and Affect Lineage Differentiation Potential and Neurite Development During Neural Differentiation of Mouse Embryonic Stem Cells

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Abstract Neuronal PAS domain protein 4 (Npas4) is a brain-specific transcription factor whose expression is enriched in neurogenic regions of the brain. In addition, it was demonstrated that Npas4 expression is dynamic and highly regulated during neural differentiation of embryonic stem cells (ESCs). While these findings implicate a role for Npas4 in neurogenesis, the underlying mechanisms of regulation remain unknown. Given that growing evidence suggests that microRNAs (miRNAs) play important roles in both embryonic and adult neurogenesis, we reasoned that miRNAs are good candidates for regulating Npas4 expression during neural differentiation of ESCs. In this study, we utilized the small RNA sequencing method to profile miRNA expression during neural differentiation of mouse ESCs. Two differentially expressed miRNAs were identified to be able to significantly reduce reporter gene activity by targeting the *Npas4* 3'UTR, namely miR-744 and miR-224. More importantly, ectopic expression of these miRNAs during neural differentiation resulted in downregulation of endogenous Npas4 expression. Subsequent functional analysis revealed that overexpression of either miR-744 or miR-224 delayed early neural differentiation, reduced GABAergic neuron production and inhibited neurite outgrowth. Collectively, our findings indicate that Npas4 not only functions at the early stages of neural differentiation but may also, in part, contribute to neuronal subtype specification and neurite development.

Introduction

Npas4 is an activity-dependent transcription factor belonging to the basic Helix-Loop-Helix (bHLH)-PAS family of regulatory proteins [1–3]. Studies have shown that bHLH-PAS proteins play important roles in a vast variety of biological processes such as embryonic development, cellular response to environmental stresses and circadian rhythm [4]. In the adult organism, Npas4 expression is largely restricted to the brain [2, 5, 6], where it is expressed in both excitatory and inhibitory neurons in response to neuronal activity-mediated calcium signaling [7–10]. Npas4 has been demonstrated to control the development of both inhibitory and excitatory synapse formation by its ability to regulate neuronal cell type-specific transcriptional programs and is therefore thought to play a central role in the homeostasis of neuronal excitation and inhibition [7, 9, 11]. Interestingly, there is mounting evidence to suggest that Npas4 may also be involved in the process of neurogenesis. Firstly, in the resting (i.e., non-stimulated) state, Npas4 expression is enriched in neurogenic regions of the brain such as the dentate gyrus of the hippocampus and the olfactory bulb [1–3, 6]. Secondly, Npas4 directly regulates the expression of brain-derived neurotrophic factor (BDNF) [7, 12], a neurotrophin that is vital for many neurobiological

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processes, including neurogenesis [13–15]. Finally, using two independent in vitro models of neurogenesis, we recently reported that Npas4 is transiently upregulated during neural differentiation of ESCs [16]. The dynamic and highly regulated pattern of Npas4 expression seen in these differentiation systems suggests a need for precise temporal control of Npas4 expression during the process of neural differentiation. However, little is known about the molecular players that regulate Npas4 expression during this process.

In the past few years, several distinct classes of small non-coding regulatory RNAs have been discovered in the brain and have been shown to play diverse roles in normal brain development and function [17]. Among them, miRNAs are the best studied and are likely to be key regulators of post-transcriptional gene expression [18]. Structurally, mature miRNAs are single-stranded RNA molecules of about 22 nucleotides in length that generally bind to the 3' untranslated region (UTR) of target mRNAs and inhibit protein synthesis either by repressing mRNA translation or by inducing target mRNA destabilization [19]. Functional studies revealed that miRNAs participate in the regulation of almost every cellular process investigated and that aberrant changes in their expression are often associated with human diseases, including cancer [20, 21]. These findings are not surprising given that bioinformatic tools predicted that each miRNA can potentially target up to hundreds of mRNAs and that together they are estimated to control the activity of more than 30 % of all protein-coding genes [22]. More importantly, accumulating evidence suggests that these miRNAs also play an important role in neurogenesis [23]. For example, it was reported that, in vitro, both miR-124 and miR-9 expression levels increased sharply during the transition from neural progenitor cells to neurons in retinoic acid-induced neural differentiation of mouse embryonal carcinoma P19 cells. Overexpression of both miRNAs was shown to promote neuronal differentiation, whereas downregulation of them had the opposite effect [24]. Interestingly, it was found that miR-9 could target the repressor element 1 (RE1)-silencing transcription factor (REST, also known as neuron-restrictive silencer factor, NRSF), which represses transcription of neuronal genes in non-neuronal tissues [25]. On the other hand, miR-124 was identified to be an important regulator of the temporal progression of adult subventricular zone neurogenesis by repressing SRY (sex determining region Y)-box 9 (Sox9) [26]. Furthermore, a subset of miRNAs (including the conserved miR-200 family which is enriched in olfactory tissues) was also demonstrated to be important for olfactory neurogenesis [27].

During central nervous system (CNS) development, neurogenesis requires precisely regulated patterns of gene expression in which positive and negative signals must be balanced to generate the correct cell types at the appropriate developmental time [28, 29]. It is now well known that miRNAs show cell- and tissue-specific as well as spatial- and temporal-

specific expression profiles [28, 30, 31], and thus, they are good candidates to regulate cellular processes that require a very fine-tuning of gene expression. Given the fact that it has been shown that miRNAs are capable of targeting Npas4 3'UTR [32, 33], we reasoned that miRNAs may be responsible for the regulation of Npas4 expression during neural differentiation of ESCs. Therefore, in the present study, we sought to test this hypothesis by searching for miRNAs that regulate Npas4 expression during neural differentiation of mouse ESCs (mESCs) and, consequently, play a role in neurogenesis.

Materials and Methods

Maintenance and Differentiation of mESCs

The cell line used for this study was 46C mESCs [34]. Routine culture of mESCs has been described previously [35]. The monolayer neural differentiation was performed as previously described [36]. Briefly, mESCs were plated onto 0.1 % gelatin-coated dishes in serum-free N2B27 medium at a density of 1×10^4 cells/cm^2. Cells were cultured at 37 °C in a humidified 5 % CO_2 atm and N2B27 medium was replaced every 2 days.

Small RNA Library Construction and Sequencing

Total RNA from each differentiation time point was isolated using the mirVana miRNA Isolation Kit (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and integrity of the RNA samples collected were confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Small RNA libraries were prepared using the TruSeq Small RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. Briefly, 3′ and 5′ adapters were sequentially ligated to small RNAs that have a 5' phosphate and a 3' hydroxyl group (from 1 μg of total RNA), followed by reverse transcription reaction using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) to generate single-stranded cDNA. The cDNA was then PCR-amplified and barcoded using a common primer and a primer containing an index tag to allow sample multiplexing. The amplified libraries were size-selected/gel-purified using the Pippin Prep System (Sage Science, Beverly, MA, USA) and quantified using the Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA). Six to eight barcoded libraries were pooled in equimolar amounts (10 nmol/L) and diluted to 8 pmol/L for cluster formation on a single flow cell lane, followed by single-end sequencing on an Illumina HiSeq 2000 sequencer. A total of 14 small RNA libraries were sequenced using two HiSeq 2000 lanes.

Small RNA Sequencing Data Analysis

Sequencing data from samples pooled in the same flow cell lane were separated (de multiplexed) using CASAVA 1.6 software (Illumina). Reads obtained were trimmed for adaptor sequences using Cutadapt v1.3 [37] with the following parameters: error rate of 20 %, overlap of 2 bases and discarding reads of shorter than 18 bases and quality below 28. Filtered reads were then mapped to the UCSC mm10 mouse genome (allowing at most three alignments) and miRBase v18 [38] (allowing up to two mismatches) using BWA aln version 0.7.5a [39]. The sequences matching known miRNAs were clustered and counted using HTSeq-count v0.5.3p9 [40]. Differential expression of miRNAs between each differentiation time point was calculated using edgeR [41].

Validation of miRNA Expression

Individual TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) were performed according to the manufacturer's instructions. Briefly, 100 ng of total RNA was reverse transcribed using the MicroRNA Reverse Transcription Kit (Applied Biosystems) with 3 μL of specific miRNA assay RT primer in a reaction volume of 15 μL. cDNA was diluted and set up in quadruplicate reactions in 96-well plates containing 0.5 μL of specific TaqMan miRNA assay (10 μL reaction) using the Bio-Rad CFX Connect Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) using the manufacturer's recommended cycling conditions. Data were analyzed with Bio-Rad CFX Manager software 2.1 (Bio-Rad Laboratories) using the comparative Ct ($\Delta\Delta$Ct) method with U6 snRNA as the endogenous control.

Luciferase Reporter Constructs

The 3'UTR of *Npas4* was PCR-amplified from the *Npas4*-minigene construct (a kind gift from Prof. Yingxi Lin) with Platinum PCR SuperMix High Fidelity (Invitrogen), using primers that introduced an *Xba*I restriction site at both the 5' and 3' ends. The resulting PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), *Xba*I digested and cloned into the *Xba*I site located downstream of the Renilla Luciferase (RL) gene in a CMV-driven RL reporter (RL-control; a kind gift from Prof. Greg Goodall) to generate the RL-*Npas4* construct. Mutant reporter plasmids were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), with the RL-*Npas4* construct as template and the primers carrying the desired mutations. The authenticity and orientation of the insert as well as the presence of the mutations were confirmed by DNA sequencing. All primers used are listed in Supplementary Table S1.

Dual Luciferase Assay

HEK 293T cells were seeded at 1.0×10^5 cells/well in 24-well plates and co-transfected 24 h later with the RL reporter constructs described above or the empty RL-control vector (5 ng), the Firefly reporter plasmid pCI-FL (5 ng; a kind gift from Dr. Kirk Jensen) and the appropriate miRNA mimics (20 nM; Ambion) using Lipofectamine 2000 (Invitrogen). Renilla and Firefly luciferase activities were measured 24 h after transfection using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA). Relative reporter activity was obtained by normalization to the Firefly luciferase activity. In order to correct for vector-dependent unspecific effects, each relative reporter activity was normalized to the empty vector co-transfected with the corresponding miRNA mimic. Results were then compared to the control mimic.

Lentivirus Production and Cell Transduction

To stably express miRNAs, the BLOCK-iT Lentiviral Pol II miR RNAi Expression System with EmGFP (Invitrogen) was used following the manufacturer's instructions. Briefly, DNA oligonucleotides encoding mmu-miR-744 and mmu-miR-224 were designed as shown in Supplementary Table S1. The DNA oligonucleotides were annealed and the double-stranded oligonucleotides were ligated separately into the pcDNA6.2-GW/± EmGFP-miR vector. The miR-744 and miR-224 expression plasmids were linearized using *Eag*I restriction enzyme, and the pDONR 221 vector was used as an intermediate to transfer the pre-miRNA expression cassette or the negative control plasmid (containing a sequence that is processed into a mature miRNA but does not target any known vertebrate genes) into the lentiviral expression plasmid (pLenti6/V5-DEST) using the Gateway Technology (Invitrogen). Subsequently, the miR-744 or miR-224 expression vector and the ViraPower Packaging Mix (Invitrogen) were co-transfected using Lipofectamine 2000 (Invitrogen) into the HEK 293FT cell line to produce a lentiviral stock. At 48 h post-transfection, the virus-containing supernatant was harvested by collecting the cell medium and passing through a 0.45 μm low protein-binding filter (Millipore, Billerica, MA, USA) to remove any non-adherent cells. The viral particles were then concentrated by ultracentrifugation. To generate stable miR-744- or miR-224-overexpressing mESC lines, 46C mESCs were transduced and cultured in a selection medium containing 4 μg/mL of blasticidin (Invitrogen) for 12 days. Control (Ctrl) mESC line was created using the negative control plasmid to control for the transduction process and for the introduction of foreign DNA into cells.

Western Blot Analysis

Cells were lysed using lysis buffer (150 mM NaCl, 1 % NP-40, 0.1 % sodium dodecyl sulfate (SDS), 1 % sodium deoxycholate and 50 mM Tris–HCl pH 8.0) containing protease inhibitor cocktail (Roche, Mannheim, Germany). Approximately 40 μg of total protein samples were loaded on 10 % SDS polyacrylamide gels and transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad Laboratories). The membranes were then blocked and incubated overnight at 4 °C with one of the following primary antibodies diluted in 0.1 % PBST: rabbit anti-β-actin (1/5000; Sigma, St. Louis, MO, USA) or rabbit anti-Npas4 (1/15,000; a kind gift from Prof. Yingxi Lin). After washing, blots were probed with the appropriate fluorophore-labeled secondary antibody for 1 h at room temperature, washed again and finally scanned on an Odyssey Infrared Scanner (LI-COR Biosciences, Lincoln, NE, USA). Where two bands were detected, both bands were included in the densitometry analysis.

Quantitative Real-Time PCR

First-strand cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) primed with 10 mM oligo(dT) and random primers (Promega, Madison, WI, USA). Quantitative real-time PCR (qRT-PCR) was performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with 50 ng of template cDNA for relative quantification of gene expression. Reactions were performed on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using 384-well plates. Cycling parameters were 95 °C for 10 min, 40 amplification cycles at 95 °C for 10 s and at 60 °C for 1 min, followed by melting curve analysis. Data were analyzed with QuantStudio software v1.1 (Applied Biosystems) using the $\Delta\Delta$Ct method. *β-actin* and *HPRT* were included as reference genes. qRT-PCR primers are list in Supplementary Table S1.

Immunocytochemistry

Cells were fixed in 4 % paraformaldehyde (PFA) in PBS for 15 min at room temperature, followed by washing twice with PBS. Cells were permeabilized with PBS/0.3 % Triton X-100 (Sigma) and non-specific binding sites were blocked with 5 % normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) in 0.1 % PBST for 1 h at room temperature. Cells were then incubated overnight at 4 °C with one of the following primary antibodies diluted in blocking buffer: mouse anti-β-III tubulin (1 in 500; Millipore), rabbit anti-vesicular glutamate transporter 1 (VGluT1, 1 in 1500; Abcam) or rabbit anti-glutamate decarboxylase 65 and 67

(GAD65/67, 1 in 1000; Abcam). After washing in 0.1 % PBST, cells were incubated with DyLight488-conjugated donkey anti-mouse IgG (1 in 300; Jackson ImmunoResearch) or Cy3-conjugated donkey anti-rabbit IgG (1 in 500; Jackson ImmunoResearch) for 2 h at room temperature. Following washing, cells were then incubated in 300 nM 4′,6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature after which they were washed twice and imaged with an Eclipse Ni microscope (Nikon, Tokyo, Japan). For quantification of VGluT1- and GAD65/67-positive cells, at least four random non-overlapping fields of view were selected at 400× magnification.

Neurite Measurement

The length of β-III tubulin-positive neurites was measured using the ImageJ plugin NeuriteTracer [42]. To obtain the mean neurite length per cell, the total neurite length per field was divided by the number of nuclei present in that field (as determined by DAPI staining).

Statistical Analysis

Results are presented as means ± standard deviation (SD). Statistical significance was assessed with one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. P-value <0.05 was considered to be statistically significant. All analyses were performed using Prism 6.0 (GraphPad Software, San Diego, CA, USA).

Results

Identification of miRNAs Involved in Regulating Npas4 Expression During Neural Differentiation of mESCs Using Small RNA Sequencing

In this study, we adopted an in vitro model of neurogenesis, in which mESCs can be differentiated toward a neural fate using the serum-free medium N2B27 [34]. We reproducibly observed that both Npas4 protein and mRNA followed a biphasic expression pattern with two peaks, one at day 4 and one at day 6 (Fig. 1a). In order to identify miRNAs that regulate Npas4 expression during neurogenesis, we performed small RNA sequencing (small RNA-seq) using total RNA isolated from undifferentiated (day 0) and differentiating (days 4, 5, 6 and 9) 46C mESCs as we expected the miRNAs to be differentially expressed between these days. Approximately 333 million (333,697,063) reads were generated by deep sequencing using the Illumina HiSeq 2000 platform. To identify high-quality reads that were considered for further analysis, raw reads were preprocessed and trimmed to remove adapter
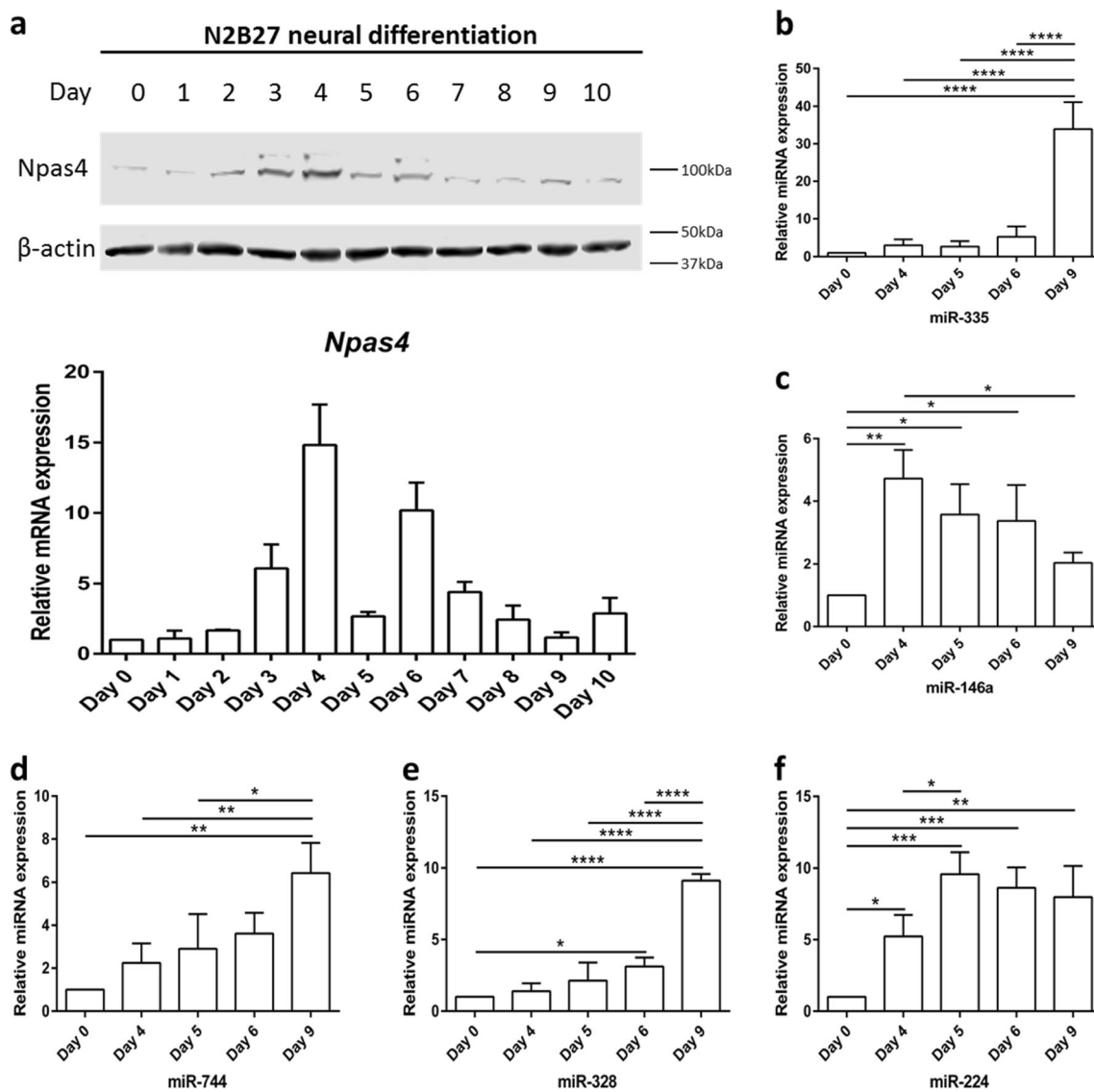
Fig. 1 Npas4 and miRNA expression profiles during neural differentiation of 46C mESCs. **a** Representative Western blot analysis of Npas4 protein expression during N2B27 differentiation of mESCs. β-actin was used as a loading control. qRT-PCR analysis of *Npas4* mRNA expression (normalized to *β-actin*) during neural differentiation. *Npas4* fold changes are relative to day 0 (undifferentiated mESCs). Values are means ± SD ($n = 4$). **b–f** Validation of five differentially expressed miRNAs detected by small RNA-seq that are predicted to regulate *Npas4* using TaqMan miRNA assays. U6 snRNA was used as a normalization control. miRNA expression levels are relative to day 0. Values are means ± SD ($n = 3$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

sequences (Supplementary Table S2). Reads were subsequently mapped to identify 146,352,275 reads that matched sequences in the mouse genome and miRBase v18 (Supplementary Table S3) and the composition of the libraries is shown in Supplementary Table S4. Currently, more than 2000 miRNAs have been identified in the mouse genome, of which around 650 have reads mapped to them in each sample.

To determine which of the approximately 650 miRNAs detected from small RNA-seq are involved in regulating Npas4, we compared these miRNAs with *Npas4*-targeting miRNAs predicted by different bioinformatic databases (TargetScan, PicTar, miRanda and MicroCosm). Given that each miRNA target prediction algorithm has its own

advantages, miRNA binding sites that are theoretically predicted by multiple bioinformatic software programs and are well conserved across mammalian species are more likely to be true binding sites. Therefore, we used these bioinformatic tools and selected miRNAs that were predicted by at least two databases, conserved across mouse, rat and human, and, more importantly, detected in our sequencing data. Based on these criteria, five miRNAs that were differentially expressed between two or more time points were chosen for further investigation, namely miR-335, miR-146a, miR-744, miR-328 and miR-224. Differential expression of these five selected miRNA candidates during neural differentiation was validated using TaqMan miRNA assays (Fig. 1b-f).

Experimental Validation of Candidate miRNAs Involved in *Npas4* 3'UTR Regulation

To evaluate whether the candidate miRNAs target the 3'UTR of *Npas4*, we cloned the 713 base pair *Npas4* 3'UTR downstream of the RL reporter gene (Fig. 2a) and co-transfected this construct into HEK 293T cells along with the five candidate miRNA mimics or the negative mimic control (miR-Neg). Interestingly, only miR-744 and miR-224 were able to significantly suppress luciferase activity as compared to miR-Neg with a corresponding decrease of about 19.08 % ($p < 0.01$) and 18.86 % ($p < 0.01$), respectively, as shown in Fig. 2b.

Notably, when both miR-744 and miR-224 were co-transfected, a synergistic effect between miRNAs was observed, as highlighted by the stronger reduction in the reporter activity of approximately 34.96 % ($p < 0.0001$). Next, to verify that the effect of the miRNAs is due to their direct interaction with the binding sites in the 3'UTR of *Npas4*, we performed site-directed mutagenesis to generate constructs in which the seed regions (miRNA recognition sequences) were mutated (Fig. 2a). Upon transfection of the miR-744 mutant construct, the downregulation of luciferase activity by miR-744 was completely rescued, demonstrating the validity of this binding site for interaction. As there are three miR-224 binding regions,
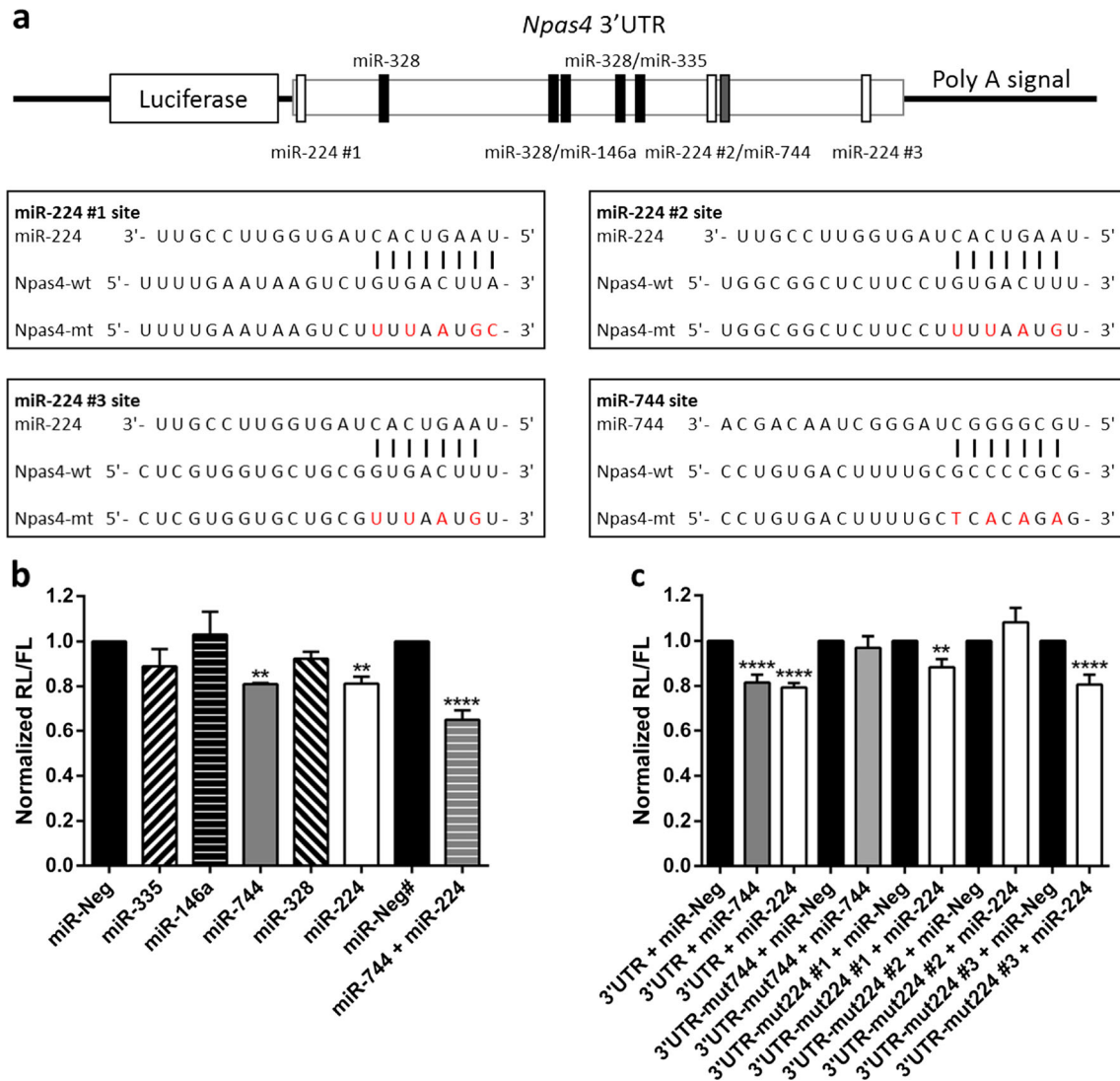
Fig. 2 miRNA-mediated suppression of reporter gene expression via interaction with the *Npas4* 3'UTR. **a** Schematic representation of *Npas4* 3'UTR in the Renilla luciferase (RL) reporter construct and the predicted miRNA binding sites within the UTR sequence. The mutated derivatives (mut744, mut224 #1, mut224 #2 and mut224 #3) were generated by introducing mutations into the sequence as shown in red. **b** HEK 293T cells were co-transfected with the RL-control (empty vector) or the RL-*Npas4* construct and the indicated miRNA mimics (20 nM). At 24 h post-transfection, luciferase activities were measured and normalized to the Firefly control. Data are presented as the normalized activity of the indicated miRNA-transfected cells relative to cells transfected with the negative mimic control (miR-Neg). Values are means ± SD ($n = 3$). **$p < 0.01$ vs miR-Neg (20 nM); ****$p < 0.0001$ vs miR-Neg# (40 nM). **c** Relative luciferase activity in HEK 293T cells co-transfected with *Npas4* wild-type 3'UTR vector or its mutant derivatives along with the appropriate miRNA mimics. Data are presented as the normalized activity of miR-transfected cells relative to cells transfected with miR-Neg. Values are means ± SD ($n = 3$). **$p < 0.01$; ****$p < 0.0001$ vs miR-Neg (20 nM)

we mutated each binding site individually and found that the miR-224 #1 and miR-224 #2 binding sites, but not the miR-224 #3 binding site, were required for the observed suppression of luciferase activity by miR-224 (Fig. 2c).

Generation of Stable miRNA Overexpressing mESC Lines and Knockdown of Endogenous Npas4 Expression

In order to investigate whether miR-744 and miR-224 down-regulate endogenous Npas4 expression during neural differentiation of mESCs, we generated several stable mESC clones that ectopically express either miR-744 or miR-224 using a lentiviral vector. For each miRNA, we generated two independent clonal cell lines to account for possible "off-target" integration effects caused by random insertion of the constructs into the mESC genome. Western blot analysis showed that endogenous Npas4 protein levels were significantly reduced at day 4 of differentiation upon miR-744 (744 #1: 67.39 %, $p < 0.0001$ and 744 #2: 87.19 %, $p < 0.0001$) or miR-224 (224 #1: mean 66.17 %, $p < 0.0001$ and 224 #2: mean 91.97 %, $p < 0.0001$) overexpression in a dosage-dependent manner (744 #1 vs 744 #2 and miR-224 #1 vs miR-224 #2: $p < 0.01$) (Fig. 3a). Overexpression of the candidate miRNAs was verified by TaqMan miRNA assays (Fig. 3b, c). The larger

fold change observed in miR-224-overexpressing clones (66.82- and 129.26-fold increase) as compared to miR-744-overexpressing clones (9.29- and 27.05-fold increase) was due to the lower basal levels of miR-224 than miR-744 in Ctrl mESCs (Supplementary Fig. S1). We also confirmed that the lentiviral transduction did not affect the pluripotency of undifferentiated mESCs (Supplementary Fig. S2). Interestingly, there was no significant difference in *Npas4* mRNA expression between the different mESC lines (Supplementary Fig. S3).

Ectopic Expression of miR-744 or miR-224 Attenuates Early Neural Differentiation of mESCs

To assess how overexpression of miR-744 or miR-224 affects neural differentiation of mESCs, we utilized gene profiling to analyze the expression of several key marker genes whose expression pattern in this model system has been well characterized and is known to define important transition events in the differentiation pathway. Using qRT-PCR, we observed that both miR-744- and miR-224-overexpressing cultures retained higher expression levels of the pluripotency marker genes *Oct4* (744 #1: $p < 0.05$ and 744 #2: $p < 0.001$; 224 #1: $p < 0.01$ and 224 #2: $p < 0.01$) and *Nanog* (744 #1: $p < 0.05$
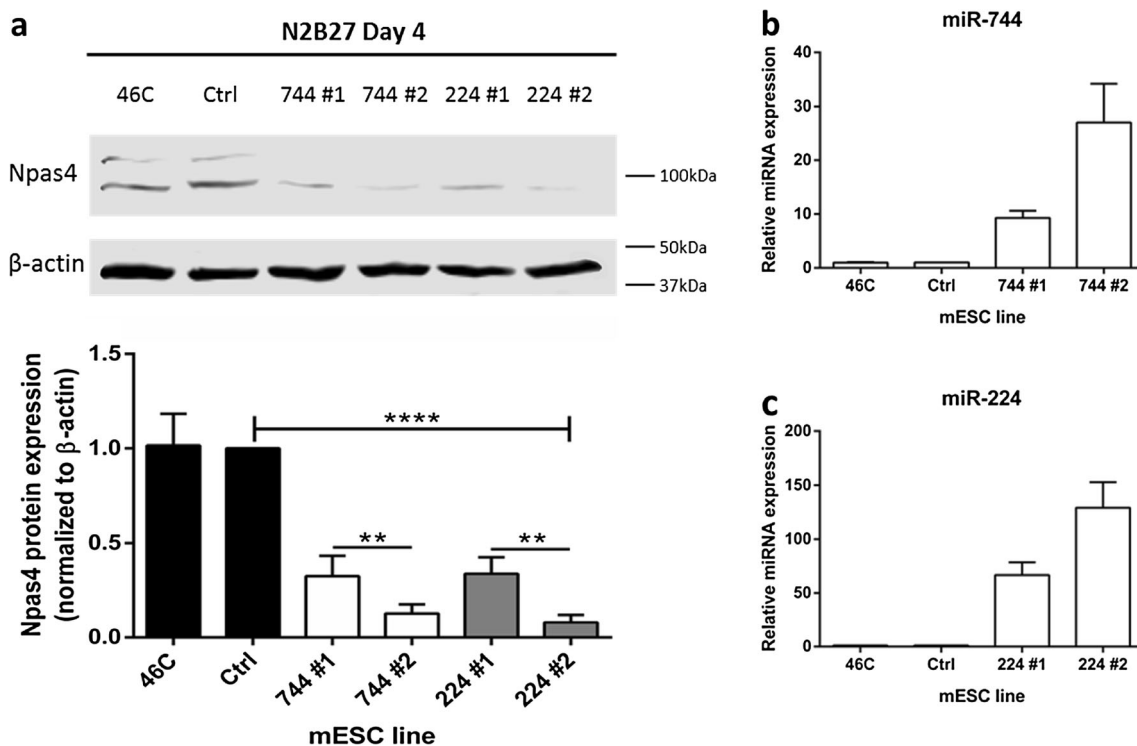
Fig. 3 Knockdown of endogenous Npas4 protein by miR-744 and miR-224 during neural differentiation of mESCs. (**a**) Representative Western blot analysis of Npas4 protein expression in different mESC lines after four days of differentiation in N2B27 medium. β-actin was used as a loading control. Quantification of Npas4 knockdown by densitometry analysis. Npas4 protein expression in each sample was normalized to β-actin expression and is relative to Npas4 expression in Ctrl line which

was given an arbitrary value of 1. Where two bands were detected, both bands were included in the densitometry analysis. Values are means ± SD ($n = 4$). **$p < 0.01$; ****$p < 0.0001$. **b**, **c** Validation of miR-744- and miR-224-overexpressing clones by TaqMan miRNA assays. miRNA expression levels are relative to Ctrl mESC line. Values are means ± SD ($n = 4$)

and 744 #2: $p < 0.05$; 224 #1: $p < 0.05$; 224 #2: $p < 0.01$) while exhibited lower expression levels of the early differentiation marker *Fgf5* (744 #1: $p < 0.01$ and 744 #2: $p < 0.01$; 224 #1: $p < 0.05$ and 224 #2: $p < 0.01$) as compared to Ctrl mESC line at day 3 of N2B27 differentiation. By day 4, expression of these genes had stabilized to control levels, indicating a lag of approximately 24 h between miRNA-overexpressing and Ctrl mESC lines (Fig. 4a–c). Similarly, we also found that expression of the neural progenitor markers *Nestin* (744 #1: $p < 0.05$ and 744 #2: $p < 0.01$; 224 #1: mean $p < 0.05$ and 224 #2: $p < 0.001$), *Pax6* (744 #1: $p < 0.05$ and 744 #2: $p < 0.01$; 224 #1: $p < 0.05$ and 224 #2: $p < 0.05$) as well as *Sox1* (744 #1: $p < 0.01$ and 744 #2: $p < 0.001$; 224 #1: $p < 0.05$ and 224 #2: $p < 0.001$) was repressed after 4 days of neural differentiation in mESCs overexpressing miR-744 or miR-224 (Fig. 4d–f). Interestingly, at day 5 when expression of these genes had dropped in uninfected and Ctrl mESC cultures, expression of *Nestin* (744 #1: $p < 0.01$ and 744 #2: $p < 0.01$; 224 #1: $p < 0.05$ and 224 #2: $p < 0.01$) and *Pax6* (744 #1: $p < 0.05$ and 744 #2: $p < 0.01$; 224 #1: $p < 0.05$ and 224 #2: $p < 0.05$) remained high in stable miRNA-overexpressing clones, further suggesting that these cultures are out of phase. Notably, these effects were not detected at a later time point (day 10; Supplementary Fig. 4).

Ectopic Expression of miR-744 or miR-224 Affects Neuronal Subtype Determination and Inhibits Neurite Outgrowth

An increasing number of studies suggest that miRNAs contribute to the neuronal diversity found in the CNS [43]. Thus, we next investigated whether overexpression of miR-744 or miR-224 has any impact on the development of specific neuronal subtypes during neural differentiation by staining mESC-derived neurons for GAD65/67 (a marker of GABAergic neurons) (Fig. 5a) and VGluT1 (a marker of glutamatergic neurons) (Fig. 6a). After 14 days in N2B27 medium, we observed that the percentage of GAD65/67-positive cells was significantly lower in cultures overexpressing either miR-744 (744 #1: 34.63 %, $p < 0.05$ and 744 #2: 23.83 %, $p < 0.01$) or miR-224 (224 #1: 35.92 %, $p < 0.05$ and 224 #2: 22.66 %, $p < 0.01$) compared to Ctrl mESCs (58.54 %) (Fig. 5b). Interestingly, the reduction in GAD65/67 expression was proportional to the level of Npas4 knockdown with the greatest reduction being observed in the cultures that had the highest level of Npas4 silencing. In contrast, overexpression of either miR-744 or miR-224 had no significant effect on the percentage of VGluT1-positive cells (Fig. 6b).

In the developing nervous system, neurite outgrowth is an important process underlying the formation of the highly
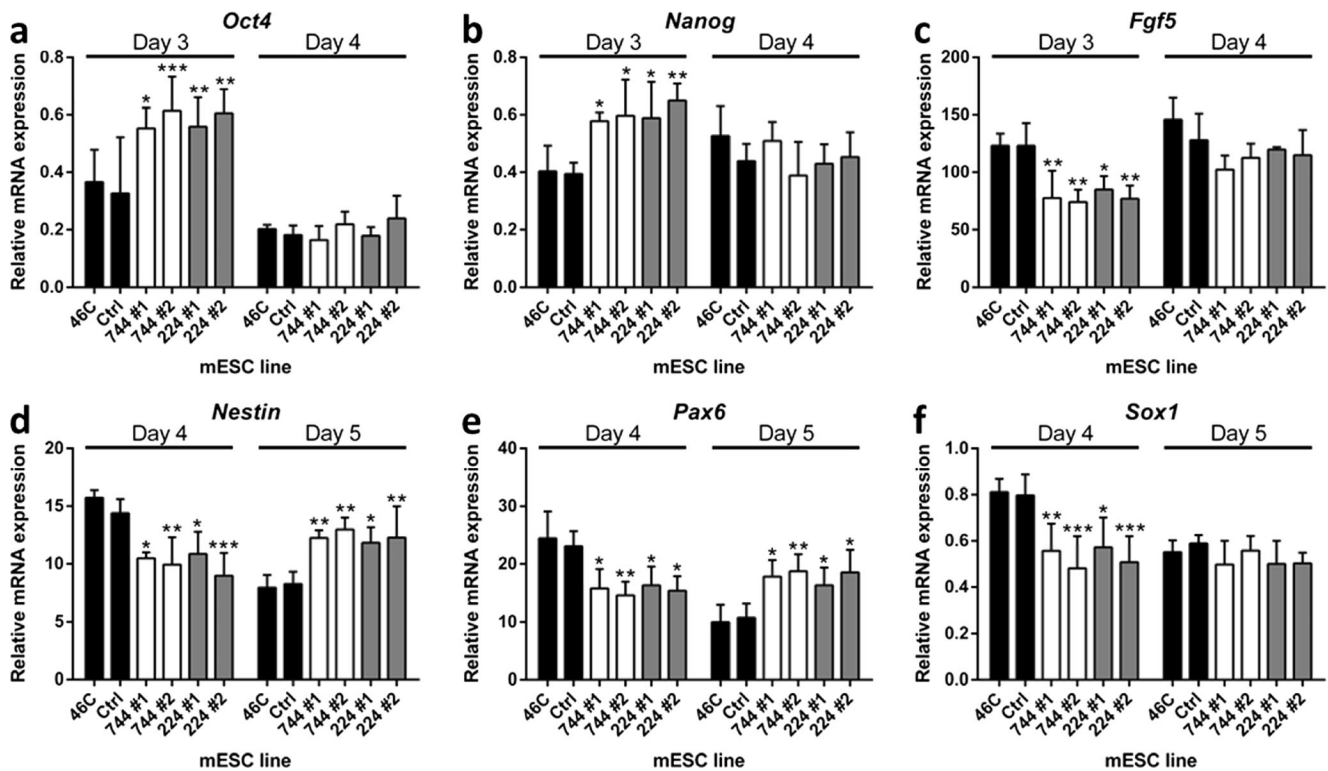
Fig. 4 Ectopic expression of miR-744 or miR-224 in mESCs impairs early neural differentiation. qRT-PCR analysis of the pluripotency markers *Oct4* (**a**) and *Nanog* (**b**), the early differentiation marker *Fgf5* (**c**) and the neural progenitor markers *Nestin* (**d**), *Pax6* (**e**) and *Sox1* (**f**) in different mESC lines cultured for 3 to 5 days in N2B27 medium. mRNA levels were normalized to *β-actin* and fold changes are relative to day 0. Values are means ± SD ($n = 4$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs Ctrl mESC line
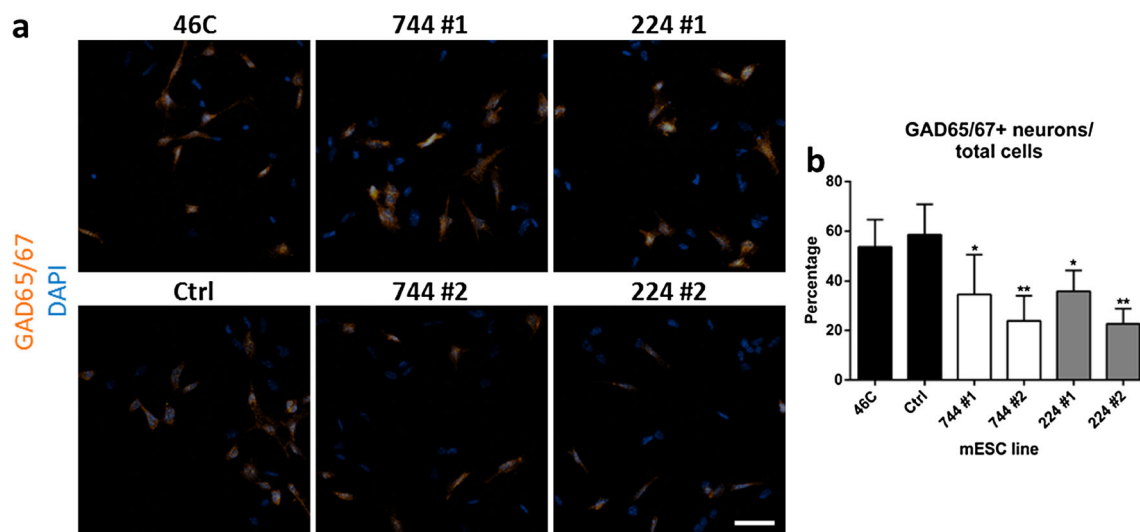
Fig. 5 Overexpression of miR-744 or miR-224 results in decreased inhibitory neuron specification. **a** Representative images of mESC-derived neuronal cultures for each mESC line immunostained with GAD65/67 (*orange*) and counterstained with DAPI to visualize nuclei (*blue*) after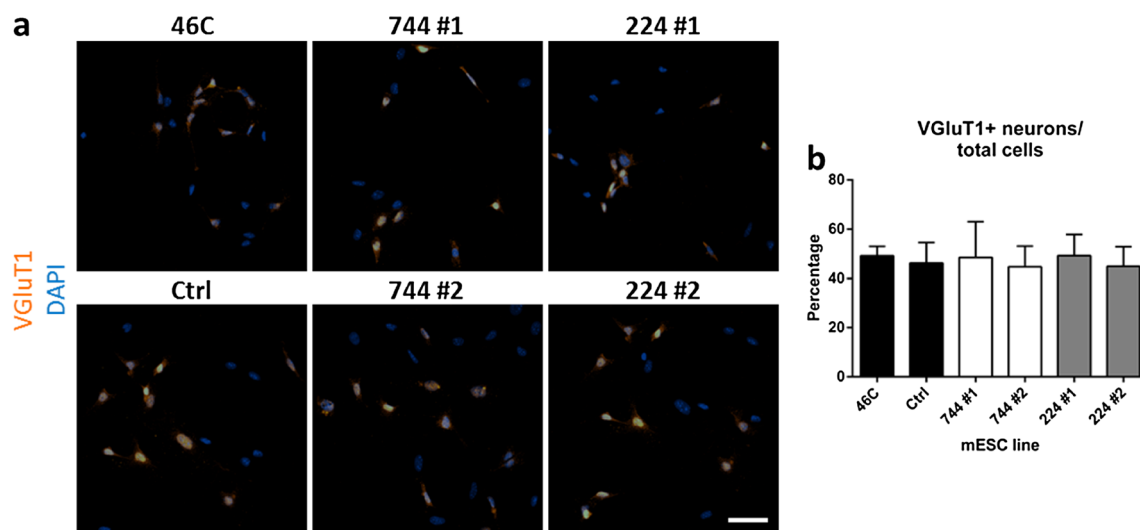 14 days of N2B27 neural differentiation. Scale bar = 50 μm. **b** Quantification of the number of GAD65/67-positive neurons relative to the total number of cells per field. Values are means ± SD ($n = 4$). *$p < 0.05$; **$p < 0.01$ vs Ctrl mESC line

specific pattern of connections between nerve cells [44]. In this study, we performed immunostaining using the anti-β-III tubulin antibody to examine the effect of miRNA overexpression on neurite outgrowth in mESC-derived neurons during neural differentiation (Fig. 7a). After 11 and 14 days of differentiation, we found significantly shorter neurites in neurons derived from miR-744- (744 #1: 65.37 μm, $p < 0.05$ and 744 #2: 31.02 μm, $p < 0.001$ for day 11 and 744 #1: 90.73 μm, $p < 0.0001$ and 744 #2: 38.54 μm, $p < 0.0001$ for day 14) and miR-224- (224 #1: 73.78 μm, $p < 0.05$ and 224 #2: 30.70 μm, $p < 0.001$ for day 11 and 224 #1: 87.71 μm, $p < 0.0001$ and 224 #2: 32.56 μm, $p < 0.0001$ for day 14) overexpressing cultures when compared with Ctrl mESC line (132.72 μm for day

11 and 182.29 μm for day 14) (Fig. 7b, c) and again, this effect was proportional to the level of Npas4 knockdown.

Discussion

Npas4 is a brain-specific bHLH-PAS transcription factor that plays a role in regulating both inhibitory and excitatory synapse formation in a neuronal cell type-specific manner [7, 9, 11]. Although we previously demonstrated that Npas4 expression is dynamic and highly regulated during neural differentiation of ESCs [16], the underlying molecular mechanisms regulating its expression in this system are still poorly

Fig. 6 Ectopic expression of miR-744 or miR-224 does not affect excitatory neuron differentiation. **a** Representative images of mESC-derived neuronal cultures for each mESC line immunostained with VGluT1 (*orange*) and counterstained with DAPI to visualize nuclei (*blue*). Images taken after 14 days of N2B27 differentiation. Scale bar = 50 μm. **b** Quantification of the number of VGluT1-positive neurons relative to the total number of cells per field. Values are means ± SD ($n = 4$)
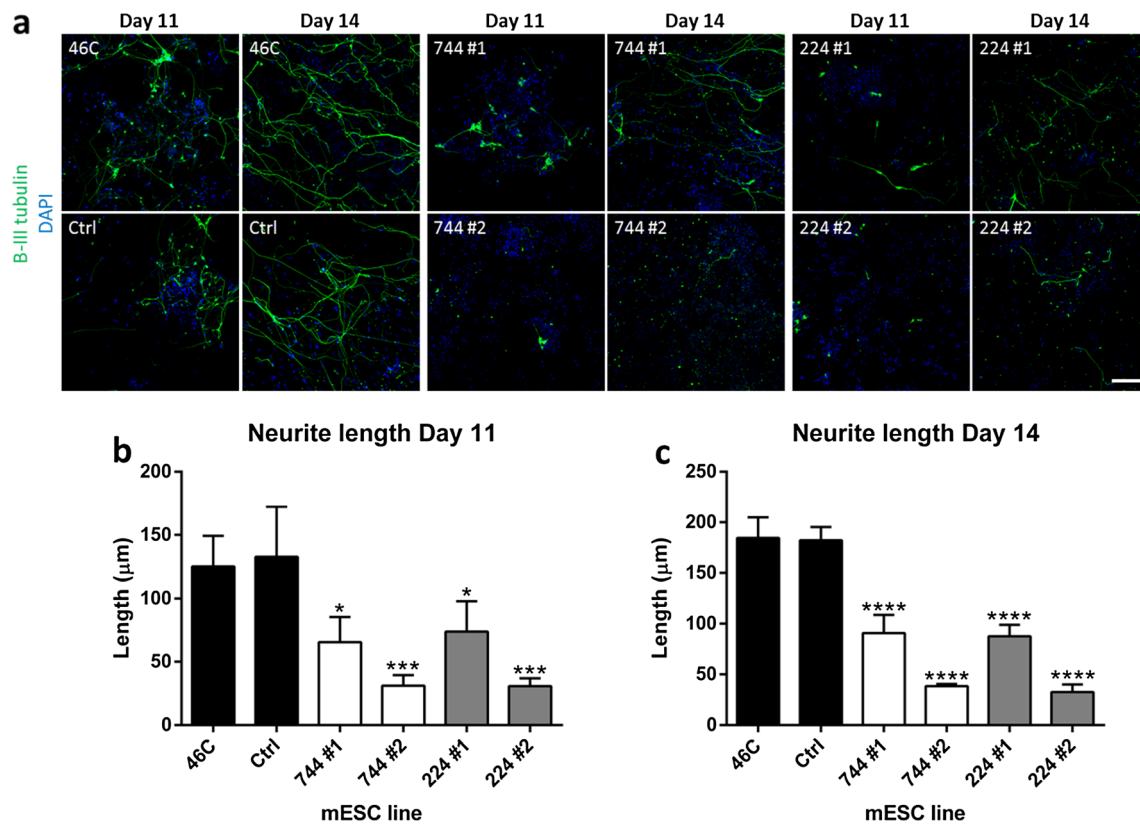
Fig. 7 Neuronal cultures derived from miR-744- and miR-224-overexpressing mESC lines show decreased neurite outgrowth. **a** Representative images of mESC-derived neuronal cultures for each mESC line after 11 and 14 days of neural differentiation in N2B27 medium. An antibody specific to the β-III tubulin protein was used to characterize neurites (*green*) and cells were counterstained with DAPI to visualize nuclei (*blue*). Scale bar = 100 μm. **b, c** Quantification of mean neurite length per cell (expressed as total neurite length divided by the number of nuclei in each field). Values are means ± SD ($n = 4$). *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$ vs Ctrl mESC line

understood. In this study, we sought to identify and investigate miRNAs that post-transcriptionally regulate the expression of Npas4 during neurogenesis by subjecting mESCs to differentiation toward the neural lineage as a monolayer using the N2B27 differentiation medium [36].

We profiled the miRNA expression during neural differentiation of mESCs using time points that correspond to the key moments in the dynamic expression pattern of Npas4 by deep sequencing. Among the miRNAs found to be differentially expressed, we selected miR-335, miR-146a, miR-744, miR-328 and miR-224 for further analysis and validated their expression profile using TaqMan miRNA assays. Interestingly, our in vitro luciferase assay revealed that only miR-744 and miR-224 were able to significantly decrease luciferase activity by targeting the 3'UTR of *Npas4* mRNA. These findings are in line with a previous study which also showed that miR-224 is capable of regulating *Npas4* 3'UTR [32]. Here, we extend upon this by demonstrating that only two of the three miR-224 binding sites present in the *Npas4* 3'UTR were required for the luciferase suppression. Several recent studies have demonstrated that cooperative interactions between miRNAs can lead to enhanced repression [45–47]. In line with these reports, when both miR-744 and miR-224 were co-transfected,

a more significant reduction of luciferase expression was observed, indicating that in this context these two miRNAs act synergistically to enhance Npas4 repression. Whether miR-744 and miR-224 also act synergistically in the event of neural differentiation remains to be seen, however, given that they peaked at a different time point during the differentiation process, this seems unlikely to be the case.

In order to assess whether miR-744 and miR-224 repress Npas4 expression during neural differentiation of mESCs, we generated several stable miR-744- or miR-224-overexpressing mESC clones. Despite the fact that a reduction of Npas4 protein expression was observed in these mESC lines, no significant difference in *Npas4* mRNA expression was detected. These data suggest that the regulation of Npas4 expression by miR-744 and miR-224 occurs specifically through suppression of protein translation rather than by mRNA degradation. We next examined the effect of miR-744 or miR-224 overexpression on the differentiation potential of mESCs and found that there was a shift in marker gene expression in miRNA-overexpressing cell lines that is indicative of delayed neural differentiation. On day 3 of differentiation, Ctrl mESCs were seen to downregulate expression of the pluripotency genes *Oct4* and *Nanog* and initiate expression of

the early differentiation marker gene *Fgf5* in preparation for neural differentiation. In stable clones overexpressing miR-744 or miR-224, this switch appeared to be delayed as the expression of *Oct4* and *Nanog* remained comparatively higher on day 3 while the expression of *Fgf5* was lower than in Ctrl cell line. By day 4, this shift was corrected, which indicates that miR-744- and miR-224-overexpressing mESC lines were approximately 24 h out of phase with Ctrl mESC line. Similarly, expression of the neural progenitor marker genes also lagged behind in miR-744- and miR-224-overexpressing cell lines as compared to Ctrl line. At day 4, expression of *Nestin*, *Pax6* and *Sox1* was higher in Ctrl mESCs, but by day 5 the expression of these genes had reached parity (*Sox1*) or was higher (*Nestin* and *Pax6*) in miRNA-overexpressing cultures, suggesting that these marker genes peak at a later time point when miR-744 or miR-224 was overexpressed. Interestingly, a similar delayed differentiation phenotype was also observed in the same differentiation system when Npas4 expression was specifically reduced using targeted small interfering RNAs (siRNAs) [16], suggesting that the knockdown of Npas4 expression is the causative factor in producing the observed phenotype.

Given that Npas4 is also expressed at early stages of neuronal development [16, 48], there is a possibility that Npas4 has a role in neuronal fate specification during neural differentiation of mESCs. Using immunostaining, we found a significant decrease in the number of inhibitory neurons in cultures derived from miR-744- or miR-224-overexpressing mESC lines. Remarkably, this effect was proportional to the level of Npas4 knockdown. Nevertheless, there was no significant influence on the number of excitatory neurons being generated. This is in accordance with our previous finding which showed that the knockdown of the zebrafish *Npas4* homolog *npas4a* resulted in a marked decrease in *dlx1a* expression, which encodes for an important regulator of GABAergic neuron differentiation [49]. Hence, the data presented suggest that Npas4 may, at least in part, be involved in the transcriptional program that controls GABAergic neuron determination. Due to its central role in neuronal excitatory/inhibitory balance, it has been suggested that Npas4 may be involved in autism. In mouse models of autism, it has been shown that autism is marked by the loss of parvalbumin-positive inhibitory neurons, which make up 40 % of the GABA cell population [50, 51]. Given the possible role of Npas4 in inhibitory neuron formation and that a greater number of mutations in the *NPAS4* gene were found in people with autism [52], it is possible that dysregulation of NPAS4 expression may affect the downstream signaling involved in autism progression.

Neurite outgrowth is a key process during neuronal differentiation and integration [53]. In this study, we found that overexpression of either miR-744 or miR-224 inhibited neurite outgrowth and that this effect was commensurate with

the level of Npas4 knockdown, indicating that Npas4 may play a role in the development of neurite outgrowth. This is consistent with studies which showed that knockdown of Npas4 inhibited neurite outgrowth in both Neuro2a cells and hippocampal neurons [54] and that *Npas4* gene dosage correlated with the degree of dendritic spine development [55]. Impaired neurite outgrowth has been associated with various neurological disorders such as schizophrenia [56] and an increasing amount of evidence also implicates a role for Npas4 in schizophrenia [57, 58]. Interestingly, miR-224 has been identified to be upregulated in schizophrenia in multiple studies [59]. Taken together with the findings presented in this study, we speculate that downregulation of Npas4 via aberrant miR-224 overexpression and a subsequent impairment of neurite outgrowth may be one of the underlying mechanisms contributing to schizophrenia.

Although miR-224 has not been extensively investigated yet, there is evidence that it has a role in stem cell differentiation. For example, it was reported that the expression of miR-224 was specifically related to generation of glial cells but not neurons during neural differentiation of adipose tissue-derived stem cells [60] and that miR-224 expression was found to be upregulated during adipogenic differentiation of mESCs [61]. Furthermore, it was demonstrated that miR-224 was highly expressed in the midbrain/hypothalamus region of the mouse brain where *Npas4* expression was shown to be low [32]. These findings, together with our data, suggest that miR-224 may be involved in regulating not only neuron-specific but also brain region-specific expression of Npas4.

Stroke is known to induce neurogenesis as a compensatory response to repair brain damage [62]. Intriguingly, miR-744 was detected to be upregulated 3 h after focal cerebral ischemia [63]. The timing of this induction corresponded well with the expression pattern of Npas4 that is seen following experimental stroke. In response to an ischemic event, Npas4 expression is rapidly upregulated and reaches a peak at 1.5 h before being downregulated at 3 h post-stroke [6, 10]. Based on our results, we hypothesize that in addition to the regulation of Npas4 during neural differentiation of mESCs, miR-744 may play a role in regulating Npas4 in the context of ischemic stroke.

It should be noted that although significant phenotypic changes were observed in this study, Npas4 is just one of the targets that miR-744 and miR-244 act upon since it has been predicted that each miRNA is able to regulate hundreds of targets due to the allowance of mismatch base pairing with mRNAs [64]. For example, miR-744 has been predicted to regulate neurogranin (NRGN), a brain-specific postsynaptic calmodulin-binding protein involved in maturation and dendritic remodeling of olfactory bulb tufted cells, during mouse postnatal development [65]. In addition, miR-224 has been predicted to regulate the expression of neuro-oncological ventral antigen 2 (NOVA2), a neuronal factor that regulates

splicing of RNAs encoding synaptic functions [66]. Thus, we were unable to exclude the possibility that knockdown of other target genes also contributed to the observed phenotypes. Nevertheless, several lines of evidence suggest that the observed effects are, at least partly, caused by a reduction in Npas4 expression. Firstly, miR-744 and miR-224 were predicted to have only two overlapping targets: Npas4 and the neuronal-specific potassium chloride co-transporter (KCC2), a key molecule in maintaining low chloride concentration in neurons [67]. Secondly, we demonstrated that expression of the Npas4 protein was reduced in stable clones overexpressing either miR-744 or miR-224. Thirdly, the severity of the observed phenotypes was concordant with the level of Npas4 knockdown in each mESC line. Fourthly, the overexpression of either miR-744 or miR-224 resulted in similar phenotypic changes. Finally, a similar phenotype was observed in the same model system when Npas4 expression was specifically reduced using siRNAs [16].

In summary, we have identified two differentially expressed miRNAs, miR-744 and miR-224, that were able to significantly reduce reporter gene activity by targeting the 3'UTR of *Npas4* mRNA. Notably, a synergistic effect between the two miRNAs was also observed. More importantly, ectopic expression of either of these miRNAs during neural differentiation of mESCs resulted in downregulation of endogenous Npas4 expression. Subsequent functional analysis showed that overexpression of miR-744 or miR-224 delayed early neural differentiation but was not sufficient to prevent commitment of mESCs to a neural fate. In addition, we have also demonstrated an effect of miR-774 or miR-224 overexpression on inhibitory but not excitatory neuron differentiation and neurite outgrowth. Interestingly, the severity of these effects was correlated with the level of Npas4 silencing. Based on these findings, we hypothesize that Npas4 plays a key role in neuronal specification and maturation during neural differentiation of mESCs. Given that neuropsychiatric diseases such as autism and schizophrenia have been associated with various neurodevelopmental abnormalities from neurogenesis to neuronal migration and integration [68], knowledge of the molecular mechanisms underlying Npas4 regulation through miRNAs may provide insight toward the development of new therapeutic strategies for the treatment of these neurodegenerative conditions.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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