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Article

Initiating astrocyte to neuron transdifferentiation via miR-124a: implications in neurodegenerative disease

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SUMMARY

Neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease, affect the lives of more than 50 million individuals each year. Neurodegeneration is the progressive damage and loss of nerve cells (neurons) in the brain. Considering the vital role that neurons play and their inability to regenerate, there is a significant challenge for the treatment of these disorders. We investigated a potential mechanism to replenish lost neurons, with the aim of restoring some cognitive and motor functions, slowing disease progression, and improving quality of life for individuals affected by neurodegenerative disorders. We hypothesized that astrocyte to neuron transdifferentiation could be initiated by the upregulation of microRNA-124a (miR-124a), causing astrocytes to go down the lineage of the specific neuronal subtype present locally. Initial *in silico* findings suggested the role of miR-124a was significant in the downregulation of polypyrimidine tract binding protein 1 (PTBP1). Additional genome analysis showed that upregulation of miR-124a was associated with enriched gene ontologies of neuron projection, axons, and genes in the synaptic vesicle pathway. To investigate these findings *in vitro*, we utilized an astrocyte cell line derived from a mouse's cerebellum (C8-D1A). After achieving a stable transfection of the cells to upregulate miR-124a, pan-neuronal markers beta tubulin III (TUJ1) and SRY-box transcription factor 2 (SOX2) showed a significant increase in expression. However, regional-specific neuronal markers were not present, suggesting that transdifferentiation began but had not progressed to full conversion. Overall, our study suggests miR-124a plays a role in initiating neuronal differentiation and should be further studied as a target in combating neurodegeneration.

INTRODUCTION

Neurodegenerative diseases are among the leading causes of disability and death worldwide, with their prevalence steadily increasing (1). Neurons play a vital role in the proper functioning of the human brain, acting as messengers that transmit sensory signals throughout the body. However, unlike the majority of cell types in the human body, nerve cells have limited to no ability to regenerate (2). Consequently, diseases that cause progressive loss of neurons, known as neurodegeneration, are very difficult to treat. Some of the most common neurodegenerative diseases include Alzheimer's disease, Parkinson's disease, and spinocerebellar ataxia (1). Alzheimer's disease is characterized by the accumulation of amyloid-beta plaques and tau tangles, leading to synaptic dysfunction, neuronal loss, and cognitive decline (3). Parkinson's disease is caused primarily by the death of dopaminergic neurons in the substantia nigra - an essential part of the basal ganglia, the area of the brain that controls movement (3). Dopaminergic neurons are nerve cells that produce an important neurotransmitter in the brain called dopamine (3). Similarly, spinocerebellar ataxias are a type of neurodegenerative disease that causes the degeneration of cerebellar Purkinje neurons (4). This causes an absence of voluntary muscle coordination, such as eye movement and speech (4).

For decades, efforts to treat neurodegenerative diseases have primarily centered on therapeutic interventions to alleviate symptoms and slow disease progression. However, currently, there are no treatments that try to reverse the natural course of this disease. Focusing on replenishing the lost neurons in the brain may be a key new approach when addressing neurodegeneration. We investigated a potential method to induce the transdifferentiation of non-neuronal cells along a neuronal lineage by investigating cellular mechanisms that inhibit or suppress the process of neuronal differentiation and countering them. Transdifferentiation is the process of making cells revert to a state where they can switch lineages and convert to a different cell type (5). It is important to note that this can easily be confused with dedifferentiation, which is when cells revert back to a less differentiated and more proliferative stage within their own lineage (5).

MicroRNAs (miRNAs) are short, non-coding RNAs that contain an average of 22 nucleotides and play an important role in regulating gene expression in cells (6). miRNAs bind to their messenger RNA (mRNA) targets, inhibiting translation, which stops protein production (7). microRNA-124a (miR-124a) is one of the most highly expressed miRNAs in the brain and has been reported to be upregulated during neurodevelopment and neurogenesis (8). miR-124a suppresses the expression of many non-neuronal genes and has been previously shown to play a role in cell fate determination, neuronal stem cell differentiation, and neuronal maturation (9). Most importantly, miR-124a is a well-known regulator of RE1-silencing transcription factor (REST), and the associated proteins that make up the REST complex. REST suppresses the expression of many neuronal-specific genes in non-neuronal cells, including miR-124a; hence, the two form a feedback loop during neuronal differentiation (10).

Another main component of this loop is polypyrimidine tract binding protein 1 (PTBP1), a protein that plays a major role in

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regulating pre-mRNA splicing (11). PTBP1 is highly expressed in non-neuronal cells but is downregulated in neurons during differentiation, which changes the alternative splicing patterns of a cell, resulting in the expression of neuronal genes (11). Therefore, we sought to investigate the miR-124a/REST loop and the role of PTBP1 in neuronal differentiation (**Figure 1**).

The central nervous system consists of a variety of cells, including neural precursor cells, neuroblasts, and glial cells, including astrocytes. Converting any of these cells into mature neurons may reduce deficits caused by neuronal degeneration. Astrocytes, non-neuronal cells that help support and protect neurons, are abundant in the brain in the same areas as lost neurons in many neurodegenerative diseases. Unlike neurons, astrocytes are able to proliferate, making them the ideal target for conversion into neurons (12).

It is important to consider the regional specificity of conversion. Throughout the brain, there are hundreds of different types of neurons, classified based on their morphology, neurotransmitter type, and function, each playing a distinct role in neural circuits. As of now, little is known about the mechanisms around what "type" of neuron a non-neuronal cell would differentiate into. Due to the distinct genetic profiles of astrocytes in various brain regions and differences in microenvironment signals, astrocytes may convert to the specific subtype of neurons naturally present around them (13). In order to assess the stage of neuronal differentiation the astrocytes reached, SRY-box transcription factor 2 (SOX2) was a significant marker in this study. SOX2 is a marker for immature neurons and neural progenitor cells, and it is indicative of the proliferative and multipotent state of these cells. However, as neuronal differentiation progresses, the downregulation of SOX2 is often observed as cells transition from a proliferative state to a more differentiated neuronal phenotype. Thus, the dynamic expression of SOX2 during neurogenesis serves as a reliable indicator of the neural stem cell population and their progression toward neuronal lineage commitment and differentiation.

We tested the hypothesis that the upregulation of miR-124a in astrocytes derived from the cerebellum would initiate the transdifferentiation of the cells into neurons. The cerebellum consists predominantly of Purkinje neurons and granule neurons. Hence, we hypothesized that if transdifferentiation was successful, it would result in neurons of one of these two subtypes. Results showed an upregulation in the expression of pan-neuronal markers, indicating a shift toward a neuronal identity. However, there was no significant change in regional specific markers. This research will contribute to a better understanding of the role and mechanisms of miR-124a in neuronal identity and regional specificity. Ultimately, insights from this study can help inform future strategies for cell-based therapies aimed at replacing lost neurons in neurodegenerative diseases.

RESULTS

First, we looked *in silico* at miR-124a's predicted target genes, obtained from an online microRNA target prediction database (14). These miR-124a target genes were put through the online analyzer EnrichR to find the enriched pathways and biological functions associated with the genes (15). Many pathways with mechanisms involving cell differentiation, axon growth, and neurite outgrowth were found to be upregulated (**Table 1**). These findings underscore the significance of

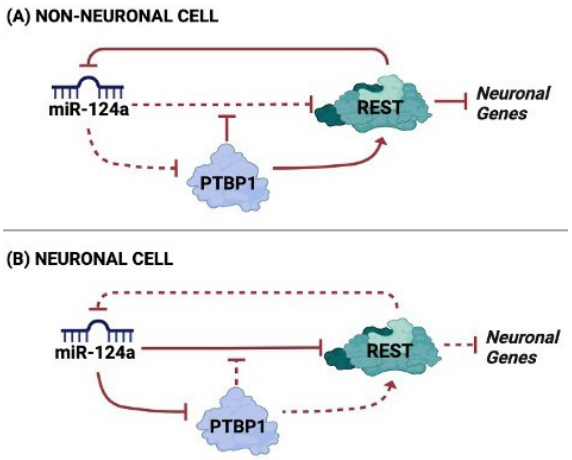

Figure 1. MicroRNA 124-1 (miR-124a) and polypyrimidine tract binding protein 1 (PTBP1) regulation of RE1-silencing transcription factor (REST) and neuronal gene expression in non-neuronal versus neuronal cells. Solid arrows represent active regulatory interactions occurring, while dotted arrows indicate pathways that are inactive. **(A)** In non-neuronal cells, high PTBP1 expression stabilizes REST activity, which inhibits neuronal gene expression. REST also suppresses miR-124a levels, reinforcing non-neuronal identity. **(B)** In neuronal cells, high miR-124a levels suppress PTBP1 expression, leading to REST inhibition and the activation of neuronal genes.

Database	Pathway	Relevant Mechanisms
PPI Hub Proteins	MAPK1 signaling pathway	cell proliferation and differentiation
BioPlanet 2019	Axon guidance	cytoskeletal dynamics, cell migration, and axon growth
BioPlanet 2019	Signaling by nerve growth factor (NGF)	cell survival, neurite outgrowth, and synaptic plasticity

Table 1. Enriched pathways among the target genes of miR-124a. Genes were put into an online analyzer Enrichr, and biological pathways that are significantly overrepresented in the input gene list were generated.

miR-124a in the regulation of various biological processes associated with cell differentiation, reaffirming its role as a key regulator and critical target for studying astrocyte to neuron transdifferentiation.

Building on the known role of miR-124a in differentiation, we investigated the impact of its overexpression in non-neuronal cells. We used a publicly available gene expression data set, GSE8498, which contains gene profiles for cath.-a-differentiated (CAD) cells overexpressing miR-124a compared to control cells. CAD cells are derived from a mouse neuroblastoma cell line. Neuroblastoma cells are not fully developed and are unable to function as normal neurons. CAD cells generally require serum starvation or retinoic acid treatment in order to further develop into mature neurons. However, this dataset was used to investigate if miR-124a upregulation was able to induce neuronal differentiation without any additional treatments (16).

Using the Gene Expression Omnibus (GEO2R) analyzer, an interactive web tool that allows for the comparison of gene expression across experimental conditions by identifying differentially expressed genes, we obtained data on the change of expression levels of various genes in the miR-124a

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treated verses control cells (17). We then placed a statistical significance threshold of an adjusted p-value (p.adj) < 0.05 and an expression change of $|\log_2(\text{Fold Change (FC)})| > 1$ to find the differentially expressed genes (DEGs) after miR-124a overexpression. We identified at total of 61 DEGs: 15 upregulated genes and 46 downregulated. Identifying the DEGs provided insight into how the overexpression of miR-124a affects and changes the gene expression profile of neuroblastoma cells. As hypothesized based on the known interactions of miR-124a and *PTBP1*, PTBP1 was significantly downregulated. This effect of miR-124a on PTBP1 activity further supports its role in regulating the inhibition of neuronal differentiation. Next, the 15 most upregulated genes were analyzed in EnrichR to look for upregulated cell types and ontologies. EnrichR gene analysis indicated a significant association with many different neuronal cell types. The most relevant and significant cell types that were enriched were trigeminal neurons, neurons, chromaffin cells, immature neurons, and interneurons (**Figure 2**). An analysis on gene ontologies showed the enrichment of neuron projections, neuron parts, cell projections, and axon ontologies (**Figure 3**). On the other hand, when the 46 downregulated genes were analyzed, cell types such as microglial cells and many astrocyte-related cells were found to be associated with the genes. This suggests a reduction in astrocyte-related gene expression, supporting the hypothesis that the cells were becoming more neuronal.

Next, we used the C8-D1A cell line, which consists of astrocytes isolated from the cerebellum of mice, and stably transfected these cells with a miR-124a expression plasmid. We saw a 1.59-fold increase of miR-124a expression in miR-124a transfected cells over those transfected with the empty control plasmid (**Table 2**). As hypothesized, an enzyme-linked immunosorbent assay (ELISA) on PTBP1 expression showed a significant decrease in the expression of PTBP1 in treated astrocytes. This supports the claim that miR-124a upregulation is not only capable of initiating neuronal maturation from precursors but also initiating transdifferentiation from astrocytes to neurons.

Pan-neuronal marker beta tubulin III (TUJ1) was found to be upregulated in the astrocytes, further indicating that the cells went down a neuronal lineage after treatment. TUJ1 is a protein that plays a crucial role in the formation of microtubules, which are essential components of the cytoskeleton (18). This protein is primarily expressed in neurons and is commonly used as a neuronal marker, especially when testing for neurodevelopment and neurobiology (18). TUJ1 is specifically associated with the early stages of neuronal differentiation and is often used as a marker to identify immature or differentiated neurons in cell cultures (18). Pan-neuronal marker and SRY-box transcription factor 2 (SOX2) was also found to be upregulated, indicating a more pluripotent state in the cells (**Figure 4**). However, while SOX2 is a marker for all neural stem cells, the concurrent upregulation of TUJ1—specific to neurons—suggests that these astrocytes are not merely reverting to a pluripotent state but are undergoing transdifferentiation toward a neuronal phenotype. However, further investigation is necessary to definitively distinguish between transdifferentiation and dedifferentiation.

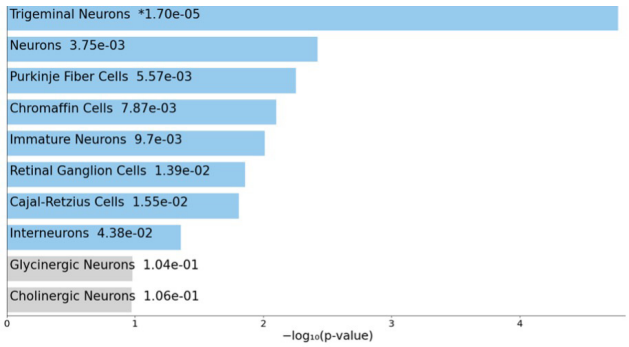

Figure 2. Top enriched cell types following miR-124a upregulation. The top 10 cell types enriched for gene signatures derived from genes upregulated in Cath.-a-differentiated (CAD) cells with increased miR-124a expression. Cell type enrichment was determined using the PanglaoDB_Augmented_2021 gene set library.

Following the initial indications of transdifferentiation to neurons, the study aimed to assess the regional specificity of this process. However, this hypothesis was not supported by the results, as zinc finger protein of cerebellum 1 (ZIC1), a granule cell-specific marker, and calbindin 1 (CALB1), a Purkinje cell specific marker, were both found to have no statically significant change in their expression levels after the upregulation of miR-124a. Further analysis was conducted to assess the presence of neurotransmitters. This was because in many neurodegenerative diseases, the primary symptoms often arise not from the direct loss of neurons but from the deficiency of specific neurotransmitters they produce within the brain (19). It was hypothesized that there would be an increase in the release of neurotransmitters. However, glutamate decarboxylase 1 (GAD1), a marker for GABAergic neurons responsible for producing the neurotransmitter gamma-aminobutyric acid (GABA), did not show any significant change in expression following the treatment. GABA was tested for as it is the main neurotransmitter in the cerebellum part of the brain (**Figure 4**).

In silico findings indicated biological processes of cell projections, axons, and neuron projections were enriched after miR-124a upregulation (**Figure 3**). Additionally, the neuronal marker TUJ1 was found to be upregulated after treatment, indicating an increase in growth of projections and axons (**Figure 4**). Next, using ImageJ, neurite growth was measured from randomly selected images of the cells with miR-124a overexpression and control cells. Six different cell projections were measured and averaged from each experimental group. A statistically significant increase in the length of cell projections in the treated group was seen ($p<0.05$) (**Figure 5**). While the projections could be either neuronal or astrocytic, the increase in TUJ1 suggests that the projections are likely neuronal.

Sample	Ct(miR-124a)	Ct(U6)	dCt
MiR-124a	10.26	10.49	-0.23
Control/Vector	12.04	11.6	0.44

Table 2. Analysis of gene expression levels after transfection. Gene expression levels were qualified using the ΔΔCq method, where the Ct values of the target gene were normalized to a reference gene and compared between experimental groups to calculate relative fold changes in gene expression. Calculation showed a 1.56 fold increase of miR-124a.

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DISCUSSION

Our results revealed the initial transdifferentiation of both neuroblastoma cells (*in silico*) and astrocytes (*in vitro*) along a neuronal lineage. Investigations began with an analysis of the predicted targets of miR-124a. The *in silico* results showed the association of the target genes with biological processes, including cell differentiation and the negative regulation of neurogenesis. Additionally, the associated pathways that are pertinent to neuronal differentiation were identified. These results underscored the potential of miR-124a as a promising target for further investigation, emphasizing its importance in the context of potential therapeutic strategies aimed to control or manipulate neuronal differentiation. To further understand the functions of miR-124a, data analysis of the DEGs from neuroblastoma cells with miR-124a overexpression versus control cells was conducted. Following the upregulation of miR-124a, numerous enriched pathways, cell types, and gene ontologies associated with neurons became evident in the cells. These results indicated that the increase in miR-124a played an important role in directing neuroblastoma cells further along the neuronal lineage (**Figure 6)**.

To further assess the efficacy of miR-124a on transdifferentiation, an astrocyte cell line was utilized in vitro. Astrocytes were chosen based on their hypothesized transdifferentiation potential and possible therapeutic significance in neurodegenerative disorders, as they are located close to the lost neurons. Results showed that expression of PTBP1, a molecule that plays a main role in inhibiting cells from going down a neuronal pathway, was downregulated. Additionally, pan-neuronal marker TUJ1 was upregulated, indicating differentiation had been initiated. However, when testing the regional specificity of conversion, specific cerebellum neuronal markers showed no statistically significant change in expression.

To assess the reason why differentiation into specific neuronal subtypes did not occur, the expression of SOX2 provides some insight. SOX2 was found to be upregulated in the cells after overexpression of miR-124a, indicating that while transdifferentiation had been initiated, it had not yet been completed to express regional neuronal types and mature neurons. All ELISAs done were performed 14 days after transfection, only showing the early and immediate changes in gene expression. It is important to note that while the *in silico* study analyzed cells incubated for only 48 hours after transfection, we extended the incubation period to 14 days to observe not only changes in gene expression but also morphological changes. Time is a contributing factor in the progression of neuronal differentiation, suggesting that the cells might not have had adequate time to fully mature and complete the differentiation process.

Investigating the short-term effects following miR-124a upregulation in astrocytes helped gain insights into the immediate molecular and cellular changes induced by miR-124a. This research was able to pinpoint the initial regulatory events and signaling pathways that are activated or suppressed in response to miR-124a overexpression. It is necessary to continue to study the role of miR-124a in neuronal differentiation and determine its long-term effects in order to apply this research to future investigations around therapies for neurodegenerative diseases. In future investigations, transfected cells will be grown for one month and then tested for expression of regional neuronal markers.

While the limited incubation time is one constraint, there are several additional limitations that need to be considered. First, the use of a monoculture system fails to capture the complex signaling and cell–cell interactions that are present in the brain. The differentiation process was likely restricted by the lack of supportive glial cells and extracellular matrix components. Additionally, the study was limited to a single astrocyte cell line and did not include any experiments on primary human cells or in vivo models, which are critical for translational relevance. In the future, these limitations could be addressed by incorporating 3D culture systems such as brain organoids or hydrogel-based scaffolds to better mimic

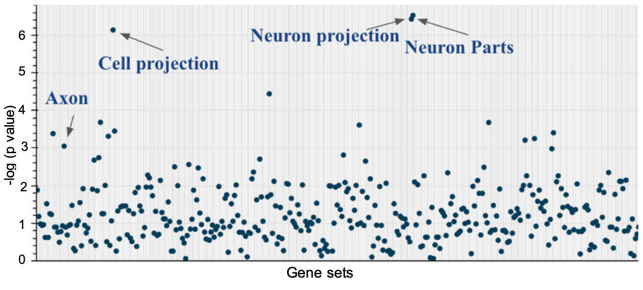

Figure 3. Enriched gene ontologies among the upregulated genes in CAD cells with increased miR-124a expression. Enriched gene ontologies are indicated with arrows and labeled. Each line on the x-axis denotes a single gene set that composes a pathway from the Jensen_COMPARTMENTS gene set. The y-axis measures the $-\log_{10}$(p-value) for each gene set that is upregulated.

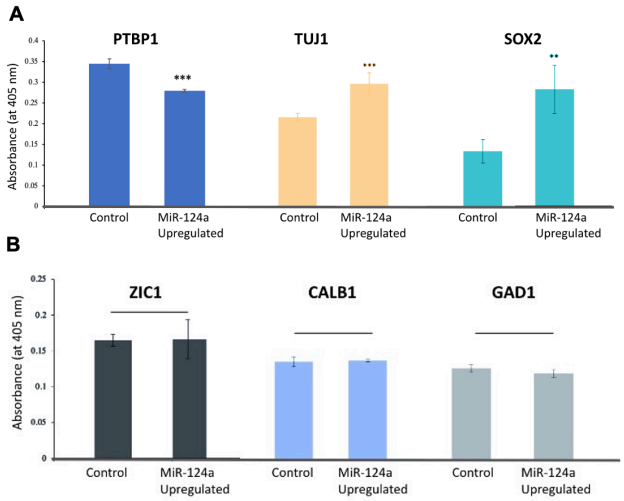

Figure 4. Expression of neuronal markers after miR-124a overexpression in C8-D1A cell line. Expression of neuronal markers was measured using an enzyme-linked immunosorbent assay (ELISA). Bars represent the mean ± standard deviation (n=4). An unpaired, two-tailed Student's t-test was conducted to assess statical significance. ** = p<0.005 ; *** = p< 0.001. **(A)** Expression level, based on absorbance, for polypyrimidine tract binding protein 1 (PTBP1, blue), beta tubulin III (TUJ1, orange) and SRY-box transcription factor 2 (SOX2, turquoise) in control cells and miR-124a upregulated cells. **(B)** Expression levels for zinc finger protein of cerebellum 1 (ZIC1, black), calbindin 1 (CALB1, light blue), and glutamate decarboxylase 1 (GAD1, grey) in control cells and miR-124a upregulated cells.

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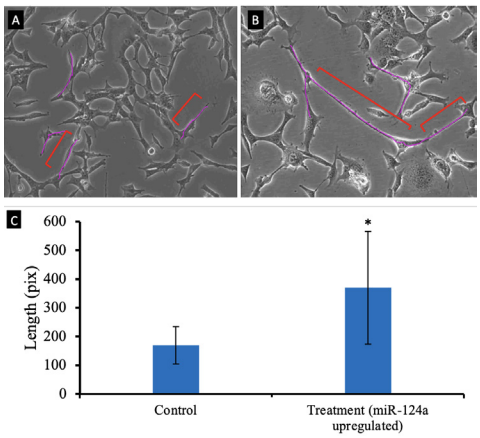

Figure 5. Neurite outgrowth after overexpression of miR-124a. **(A)** Sample image of control cells. **(B)** Sample image of treated cells after the overexpression of miR-124a. Pink lines represent traced cell projections that were quantified by length in pixels. Images taken with Nikon Eclipse TS100 microscope at 100x magnification. **(C)** Average of cell projection lengths. Bars represent means ± SD (n=6). An unpaired, two-tailed Student's t-test was conducted to assess statical significance. * = p< 0.05.

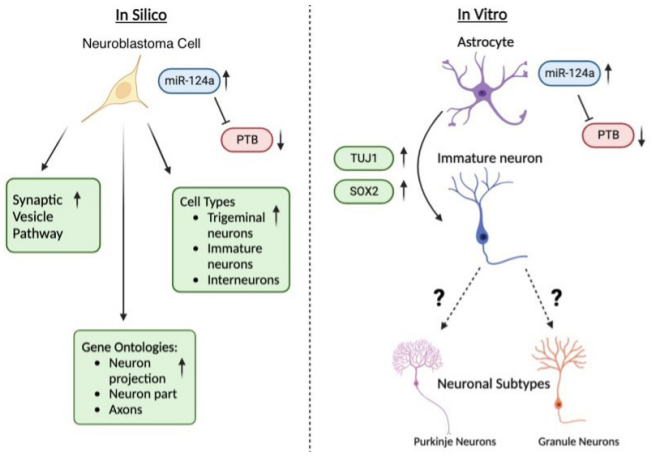

Figure 6. Summary of findings. *In silico* results showed as miR-124a increased, PTBP1 decreased and neuronal related pathways, cell types, and gene ontologies increased. *In vitro* results showed as miR-124a increased, PTBP1 decreased, pan-neuronal markers increased, and specific neuronal subtype markers showed no change.

the neural microenvironment. Co-culturing astrocytes with neurons or microglia and using brain-on-a-chip technology could also provide more physiologically relevant data.

Ultimately, these findings lay the groundwork for more comprehensive studies on miR-124a upregulation and its role in cellular reprogramming. By demonstrating the initiation of astrocyte-to-neuron transdifferentiation, this study highlights the potential of miR-124a as a molecular tool to convert resident, non-neuronal cells in the brain into neurons. This could pave the way for regenerative therapies that do not rely on stem cell transplantation or invasive procedures. miR-124a-based strategies may offer a means of replenishing lost neurons directly within affected brain regions, thereby restoring neural circuits and function in patients with neurodegenerative diseases such as Parkinson's and Alzheimer's. If optimized and validated in human models, this approach could significantly enhance our ability to treat these conditions at the cellular level, offering hope for more effective, personalized, and long-lasting interventions.

MATERIALS AND METHODS

miRDB - MicroRNA Target Prediction Database

MiRDB is an online resource that uses the MirTarget bioinformatics tool to predict miRNA targets. MirTarget uses machine learning methods to analyze numerous miRNA interactions with their targets. It does so by identifying common reactions to miRNA binding and changes in the expression of possible targets (14). It was used to identify the predicted target genes of miR-124a.

Gene Expression Omnibus (GEO)

For our analysis, we utilized the publicly available gene expression dataset GSE8498 from the Gene Expression Omnibus (GEO), originally generated by a research group studying gene regulation in CAD cells (mouse neuroblastoma cell line) transfected with a plasmid expressing miR-124a-2, alongside a control group transfected with an empty plasmid (16). In this dataset, gene expression levels were measured in untreated CAD cells and in CAD cells 48 hours after transfection. The dataset includes only expression values, which we analyzed using GEO2R, an interactive online tool designed to compare gene expression across multiple experimental conditions and quantify differential expression (17).

Enriched gene ontologies and cell types

The upregulated differentially expressed genes (DEGs) from the GSE8498 dataset were determined by an p.adj < 0.05 and an $|\log_2 FC| > 1$. The DEG's list was exported to an online tool, EnrichR, which identified significant gene ontologies, cell types, and pathways associated with the differentially expressed genes (15). For the GSE8498 data set, associated ontologies were found by comparison with the Jensen_COMPARTMENTS gene set and associated cell types were found by comparison with the PanglaoDB_Augmented_2021 gene set. The list of predicted miR-124a gene targets was compared to PPI Hub Proteins, WikiPathway 2023 Human, and BioPlanet 2019 databases to find associated pathways.

Cell culture

The cells used in experimentation were mouse (Mus musculus) astrocytes (C8-D1A) purchased from the American Type Culture Collection (ATCC). The cells were cultured at 37°C with 5% CO_2 and grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen), and 1% N_2 growth supplement (Invitrogen). When cells reached confluency, they were split and transferred to fresh medium using 0.05% trypsin-EDTA (Invitrogen). Trypan blue exclusion using a Vi-CELL XR Cell Viability Analyzer was used to assess the cells viability. Cells for all assays were plated at 0.12 x 10^6 cells/ml.

Transfection

C8-D1A astrocytes were transfected with MegaTran 1.0/DNA complexes (Origene, Rockville, MD) for miR-124a and

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PCMV-miR (empty vector) according to Origene protocol for DNA plasmid transfection. Stable transfection was maintained in 400 µg/mL of G418 (Sigma, St. Louis, MO) media. The success of transfection was confirmed through quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA was isolated from miR-124a and PCMV-miR transfected cells using Origene's Vantage RNA Kit and converted into complementary DNA (cDNA) for qRT-PCR. After qRT-PCR was performed, data was analyzed using the ΔΔCq method.

Preparation of lysates

Phosphate buffer saline (PBS) (10010023, Gibco, Waltham, Massachusetts, USA), 1X lysis buffer (9803S, Cell Signaling Technology, Danvers, MA, USA), and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) were kept on ice. Cells were trypsinized and centrifuged at 1200 rpm for 7 minutes to form a pellet. Cell pellets were resuspended with 1 mL of PBS, centrifuged in a microcentrifuge tube, and subsequently centrifuged again at 1000 rpm for 7 minutes. PBS was poured out, and 1 mL of lysis buffer (containing 1% protease inhibitor cocktail) was added at a concentration of 1 x 10^6 cells/mL. Microcentrifuge tubes were placed on ice for ten minutes. Samples were centrifuged at 13,000 rpm for 15 minutes at 4°C. Clear supernatant was decanted into microcentrifuge tubes, which were chilled on ice. Lysates and supernatants were stored at -80°C until use.

Indirect Enzyme-Linked Immunosorbent Assays (ELISA)

Indirect ELISAs were used to determine the expression of various neuronal marker proteins in cells treated with miR-124a expression plasmid. Lysates were plated at 100 microliters per well in a 96-well ELISA plate and sealed with a plastic film. After 1 hour, liquid in the plate was decanted, and 300 µL of bovine serum albumin (BSA) Blocking Buffer was administered to each well. After 1 hour, BSA Buffer was decanted, and 100 µL of the primary antibody was added in each well for one hour and incubated at room temperature. The primary antibodies used were specific for proteins PTBP1 (TA380479 , OriGene), TUJ1 (TA382932, OriGene), SOX2 (TA381896, OriGene), CALB1 (10217-654, VWR/AVANTOR), ZIC1 (200-401-159, FISHER/Thermo-scientific), and GAD1 (TA371193, OriGene). Then, the primary antibody was removed, plates were washed 3 times with a wash buffer, and 100 µL of goat anti-rabbit secondary antibody (31460, Invitrogen) was added in each well for one hour and the plate was incubated at room temperature. After washing with a wash buffer, 100 µL ABTS Substrate Solution (KPL) was added to each well. The plate was read at 405 nm in the BioTek ELx808 Absorbance Plate Reader.

ImageJ analysis

Images of cells were taken with the Nikon Eclipse TS100 microscope at 100x magnification and were randomly selected to be analyzed. Images were then uploaded to ImageJ, a public domain software for processing and analyzing scientific images. Fiji, a distribution of ImageJ that includes many useful plugins, including NeuronJ, was used to analyze morphological/cell projection changes in the cells after treatment. Six cell projections were measured and averaged for analysis.

Statistical analysis and figures

Statistical significance was determined using an unpaired, two-tailed Student's t-test. An p.adj < 0.05 was considered statistically significant. Log_2(FC) values greater than 1 or less than -1 were considered biologically relevant. Assays were conducted two or more times, each with four replicates (n = 4). Standard deviation is represented by error bars in all bar graphs. Figures and diagrams were created by the author using Microsoft Excel, BioRender, GEO2R, EnrichR, and Appyter.

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