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Metadichol® Induced the Expression of Neuronal Transcription Factors in Human Fibroblast Dermal Cells

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Abstract

Background: Producing neurons from fibroblast cells has the potential to treat neurodegenerative diseases, characterized by neuron loss. Neurodegenerative diseases are a growing problem in the current aging and developed world populations. Metadichol® is a nontoxic nanoemulsion of long-chain lipid alcohols currently available as an oral supplement.

Methods and Findings: In this study, Metadichol® was used to treat human fibroblasts in vitro; we subsequently evaluated changes in the expression of neuronal transcription factors by qRT–PCR and immunoblotting. We observed increased expression of critical transcription factors for neuronal development, such as *ASCL1 and NGN2. ND2, NR4A2, LMX1A, LHX3. ISL1,* and *FOXA2*.

Conclusions: These data suggest that Metadichol® is a promising putative neuronal remodeling agent. Its current availability and safety profile suggest that it could be rapidly available for in vivo testing, which has been impossible thus far.

Keywords: Machine learning; Disease; Autoimmune; Single cell RNA-sequencing; Drug discovery

Introduction

Approximately 10 million Americans suffer from neurogenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease), and Huntington's disease. As neurodegenerative diseases begin in mid-life, in affluent societies with a low birthrate and aging populations such as the United States, Europe, and Japan, an increased incidence of neurodegenerative diseases is expected over the coming decades. Neurodegenerative disease results from neuronal damage and death in the brain or spinal cord [1]. With progressing neuron death, patients experience increasingly debilitating symptoms such as memory loss, hand tremors, and loss of the ability to walk or manage daily self-care tasks. Unfortunately, numerous neurodegenerative diseases are eventually fatal. Tens of millions of people will be impacted by these diseases in developed countries; therefore, there is an urgent need for effective treatments against their effects.

To this end, current research focuses on ways to regenerate lost neurons. Specifically, human fibroblasts have been reprogrammed into motor neurons, cholinergic neurons, dopaminergic neurons, and others by delivery of specific transcription factors (TFs) through viral vectors. In particular, the use of the BRN2, ASCL1, MYT1L, and NEUROD1 transcription factors to reprogram fibroblasts into neurons was a breakthrough in regenerative medicine [2].

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Fibroblasts were chosen as target cells due to their relative abundance and ease of obtention through minimally invasive methods, allowing the generation of patient-specific cells. However, fibroblast cell cultures contain heterogeneous cell types and neural crest-derived stem cells [3] and multipotent stem cells with the ability to differentiate into neurons.

Using TFs to transform fibroblasts into neurons is similar to Yamanaka et al.'s approach, which involved direct conversion of mouse and human fibroblasts into induced pluripotent stem cells (iPSCs) using the transcription factors Oct3/4, Sox2, Klf4, and c-Myc (OSKM) [4,5]. Similarly, other groups promoted the conversion of fibroblasts harvested from patients with neurological disorders into iPSCs and, subsequently, into functional neurons [6-9].

TFs that control gene expression are critical during both embryonic development and aging. At the embryonic level, TFs control the differentiation of pluripotent stem cells into different tissue types with distinct biological functions [10]. Each neuronal TF has a role that establishes connections between neuronal cell types [11,12], as flawed neuronal development can lead to autism [13] or neurodegenerative diseases such as AD, Huntington's disease, or schizophrenia [14-17].

In this study, summarized in Figure 1, based on a literature review, we have listed 23 genes (Table1, 2,3) that are involved in neuronal differentiation and have a critical role in neurodegenerative diseases. We then carried out analysis using the software Pathway Studio, Elsevier [18,19], to generate a gene network and list of cellular processes involved in neurogenesis (see Figure 2, Table 4). To experimentally prove this, we treated human dermal fibroblasts with Metadichol® [20], a nanoemulsion of long-chain lipid alcohols, at various doses (1 picogram, 100 picogram, 1 nanogram, and 100 nanogram) and analyzed its effect on the expression of neuronal TFs using q-RT–PCR for quantification of expressed genes and qualification with Western blot studies. Our results showed that treating fibroblasts with Metadichol® can upregulate TFs important for neuronal differentiation.

Materials and Methods

All work was outsourced carried out by a service provider, Skanda Life Sciences (Bangalore, India).

Chemicals and reagents

Fibroblast cells were obtained from ATCC (USA). Primary antibodies were purchased from ABclonal (Woburn, MA, USA) and Elabscience (Houston, Texas USA). Primers (Table 1) were sourced from SahaGene (Hyderabad, India). Additional reagents were obtained from Sigma–Aldrich, Bangaloore, India.

Maintenance and seeding

The cells were maintained in the appropriate medium, with or without the required supplements and 1% antibiotics, in a humidified atmosphere of 5% CO2 at 37°C. The medium was changed every other day until the cells reached confluency. The viability of the cells was assessed using a hemocytometer.

When the cells reached 70%–80% confluence, single-cell suspensions containing 106 cells/mL were prepared and seeded into 6-well plates at a density of 1 million cells per well. The cells were incubated for 24 h at 37°C in 5% CO2. After 24 h, the cell monolayer was rinsed with serum-free medium and treated with Metadichol at the concentrations described below.

Cell treatments

Different concentrations of Metadichol (1 pg/mL, 100 pg/mL, 1 ng/ml, and 100 ng/mL) were prepared in serum-free medium. Subsequently, a Metadichol-containing medium was added to predesignated wells. Control cells received medium without the drug. The cells were then incubated for 24 h. After treatment, the cells were gently rinsed with sterile PBS.

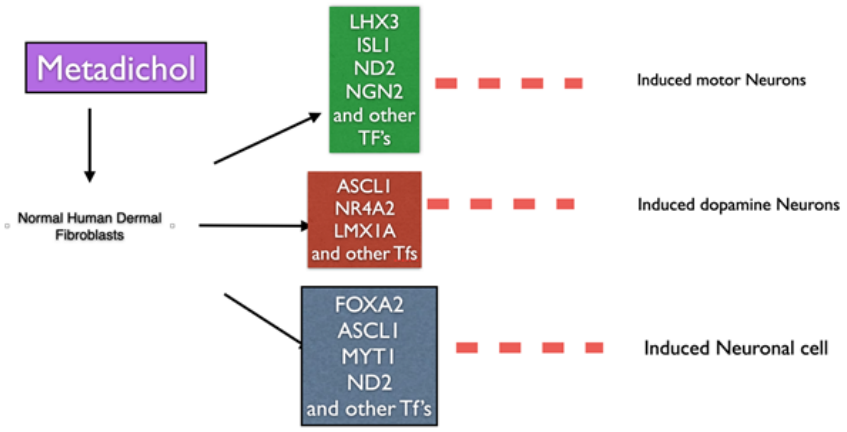

Figure 1: Expression of neuronal transcription factors

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Quantitative real-time PCR (qRT–PCR)

RNA isolation

Total RNA was isolated from each sample using TRIzol. Approximately 1×106 cells were collected in 1.5-mL microcentrifuge tubes. The cells were thereafter centrifuged at 5000 rpm for 5 min at 4°C, and the cell supernatant was discarded. Then, 650 µL of TRIzol was added to the pellet, and the contents were mixed well and incubated for 20 min on ice. Next, 300 µL of chloroform was added, and the samples were mixed for 1–2 min by gentle inversion and incubated for 10 min on ice. Then, the samples were centrifuged at 12,000 rpm for 15 min at 4°C. The upper aqueous layer was transferred into a sterile 1.5-mL centrifuge tube, an equal amount of prechilled isopropanol was added, and the samples were incubated at −20°C for 60 min. After incubation, the mixture was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was thereafter carefully discarded. The pellet containing RNA was washed with 1.0 mL of 100% ethanol, followed by 700 µL of 70% ethanol via centrifugation, as described above. The RNA pellet was air-dried at room temperature for 15–20 min. Then, it was resuspended in 30 µL of DEPC-treated water. The RNA concentration was quantified using a SpectraDrop (SpectraMax i3x, USA) spectrophotometer (Molecular Devices). Finally, cDNA was synthesized using reverse-transcription PCR (RT–PCR).

cDNA synthesis

cDNA was synthesized from 2 µg RNA using the PrimeScript cDNA synthesis kit (Takara) and oligo-dT primers according to the manufacturer's instructions. A 20 µL reaction volume was used, and cDNA synthesis was performed on an Applied Biosystems instrument (Veriti). Then, qPCR was carried out (50°C for 30 min followed by 85°C for 5 min).

Primers and qPCR

The PCR mixture (at a final volume of 20 µL) contained 1 µL of cDNA, 10 µL of SYBR Green Master Mix, and 1 µM specific forward and reverse primers for the respective target genes. PCR was performed under the following conditions: an initial denaturation at 95°C for 5 min, followed by 30 cycles of secondary denaturation at 95°C for 30 s, annealing at the optimized temperature for 30 s, and extension at 72°C for 1 min. The number of cycles amplifying in the exponential range without reaching a plateau was selected as the optimal cycle number. The results were then analyzed using CFX Maestro software. Fold change was calculated using the fold change calculated using the following equation.

(ΔΔCT Method)

The comparative CT method determined the relative expression of target genes to the housekeeping gene (β-actin) and untreated control cells.

The delta CT for each treatment was calculated using the formula.

Delta Ct = Ct (target gene) – Ct (reference gene)

To compare the delta Ct of individually treated samples with the untreated control sample, the Ct was subtracted from the control to obtain the delta delta CT.

Delta delta Ct = delta Ct (treatment group) – delta Ct (control group)

The fold change in target gene expression for each treatment was calculated using the formula. Fold change = 2^ (−delta delta Ct)

Protein isolation and Western Blots

Total protein was isolated from 106 cells with RIPA buffer supplemented with the protease inhibitor PMSF (Phenuylmethyl sulfonyl fluoride). The cells were lysed for 30 min at 4°C while gently inverted. Next, the cells were centrifuged at 10,000 rpm for 15 min. The supernatant was transferred to a new tube. The Bradford method was used to determine the protein concentration, and 25 µg of protein was mixed with 1× sample loading dye containing SDS and loaded onto a gel. Proteins were then separated in Tris-glycine buffer under denaturing conditions.

The proteins were then transferred onto methanol activated PVDF membranes (Invitrogen) using the Trans-Blot Turbo system (Bio-Rad, USA). Nonspecific binding to the membranes was blocked via incubation in 5% BSA for 1 h. The membranes were then incubated overnight with the respective primary antibodies at 4°C and then with a species-specific secondary antibody for 1 h at room temperature. The blots were washed and incubated with ECL substrate (Merck) for 1 min in the dark. The images showing the results were captured at appropriate exposure settings using the ChemiDoc XRS system (Bio-Rad, USA).

ΔΔCT method

We determined the relative expression of the target gene in relation to a housekeeping gene (β-actin) and untreated control cells by the comparative CT method. The ΔCT for each treatment was calculated using the formula:

ΔCT = CT (target gene)–CT (reference genes).

To obtain a ΔΔCT, we subtracted individual samples in the treated and control groups as follows:

ΔΔCT = ΔCT (treatment group)–ΔCT (control group).

Similarly, we calculated the fold change in target gene expression for each treatment using the formula:

Fold change = 2^ (-ΔΔCT)

Protein isolation

We isolated total protein from 106 cells using RIPA buffer supplemented with the broad-spectrum protease inhibitor phenylmethylsulfonyl fluoride. We applied a mild inversion for 30 min at 4°C to lyse the cells and then centrifuged them at

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Table 1: Primers used

Gene	SEQUENCE		SIZE	Tm
GATA-2	F	GGAACCGGAAGATGTCCAACA	169	60.27
	R	ATGTGTCCGGAGTGGCTGAA		61.48
MYT1L	F	GCAGGCAGTGATGAACAACC	201	59.76
	R	GGTTTGGGACTTGGGATGGT		59.89
GATA-3	F	TGGGCAATCAGTGTTACCGTT	309	60.2
	R	CTCCGAGCACAACCACCTT		59.93
PHOX2B	F	CCAAGGCTATTGTCGTCGCT	159	60.46
	R	TGCGAAGCCAGGGAAGTTTG		61.17
NR4A2	F	AACACCGTCCAACATTCCTTG	119	59.05
	R	GCTGCTGCATGCAAGTTTT		58.09
KLF7	F	CTTCTCTCGACGCCATCTCC	247	59.97
	R	AGCCATCCAAAAGCCCCATT		60.25
FOXA2	F	ACTCGCTCTCCTTCAACGAC	242	59.76
	R	CTCCCCGAGTTGAGCCTGTG		62.51
NEUROG2	F	CTGGGAAGAGATGATGGTGGC	106	60.48
	R	GAGATTCACACGAACTGCACC		59.54
LMX1A	F	TCTGTGTGGCTGATGGTGTT	256	59.53
	R	AAGCCTTGGTGTTTCCCAGT		59.74
ISL2	F	TGGTCTCCTTCTCCGAGTCC	106	60.32
	R	GGACTCGGCACCATACTGTT		59.75
ASCL1	F	GCGGCCAACAAGAAGATGAG	308	59.55
	R	CCAAAGTCCATTCGCACCAG		59.48
POU3F2	F	TTCTCGCTTATCTCCGTGGC	387	59.9
	R	GTTTCCGCCGTGATGTTCTG		59.8
DLX1	F	TACTTTAAGCGCACGGGGAG	103	60.11
	R	CATTCGGCTCCAAACTCTCCA		60.34
DLX2	F	AAGTTTAGGTGCCTTTGCGG	140	59.04
	R	AACTCTGTGTCCAAGTCCAGG		59.58
TP53	F	AGGTTGGCTCTGACTGTACC	332	59.02
	R	CCCACGGATCTGAAGGGTGA		61.26
LHX3	F	CGAGGGGAGAGCGTTTACTG	131	60
	RR	CAGTGCAGGTGGTACACGAA		60
BCL11B	F	TTGCCAGGACTAAGCCATCC	387	59.74
	R	TGCAGGGCTGAGTTACAAGG		59.96
HAND2	F	GACCCAGGACTCCGGAAAAG	201	60.04
	R	ACGGGAGTGTCCTCTTCGTA		59
NEUROD1	F	TCTTCCACGTTAAGCCTCCG	97	59.75
	R	CCATCAAAGGAAGGGCTGGT		59.85
NEUROG1	F	TCTTGGTCTGTTTCTCCGGC	162	59.85
	R	GGGTCAGTTCTGAGCCAGTC		60
PITX3	F	GAGCACAGCGACTCAGAAAAG	359	59.54
	R	CAGTTGCCGTACGAGTAGCC		60.8
KL	F	AGGGTCCTAGGCTGGAATGT	158	59.02
	R	CCTCAGGGACACAGGGTTTA		60
TERT	F	CCCAAGTCCCTGAACTGTGT	150	60.04
	R	ACATTGAAGGCCAAGGTACG		69

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Table 2: Fibroblast cells and reprogramming factors

Cell type	Reprogramming factors
Induced neurons	PoU3F2, ASCL1, MYTL11
human induced neurons, induced dopaminergic neurons	PoU3F2, Ascl1, MYT1L
human induced neurons, induced dopaminergic neurons	Ascll, Nurrl, LmxlA
human induced neurons	PoU3F2, Ascl1, NeuroDl, MYT1L
human induced neurons, induced dopaminergic neurons	PoU3F2, Ascl1, FoxA2, LmxlA, MYT1L
Induced motor neurons	PoU3F2, Ascl1, Lhx3, Ngn2, Isll, Ngn2, NeuroDl,MYT1L
human induced neurons, induced dopaminergic neurons	Ascll, Myt1, NeuroD2
induced neural stem cells, induced neural progenitor cells	Oct4, Sox2, Klf4, c-Myc
induced neural stem cells, induced neural progenitor cells	Sox2, Klf4, c-Myc,

Table 3: Transcription factors play a key role in neurogenesis.

Name	Description	
KLF7	Kruppel like factor 7	control neuronal morphogenesis and promotes axon outgrowth.
NR4A2	nuclear receptor subfamily 4 group A member 2	Regulates dopaminergic (DA) neuronal differentiation, survival, and maintenance
DLX1	distal-less homeobox 1	Increases Interneuron GABA Synthesis, Synaptogenesis, and Dendritogenesis
HAND2	heart and neural crest derivatives expressed 2	Needed for neurogenesis and subset of cell type-specific markers in the development of enteric nervous system
MYT1L	myelin transcription factor 1 like	dysfunction results in Neuro disorders.
DLX2	distal-less homeobox 2	Increases Interneuron GABA Synthesis, Synaptogenesis, and Dendritogenesis
BCL11B	BAF chromatin remodeling complex subunit BCL11B	regulate progenitor cell proliferation differentiation, migration, and carry out integration of neural cells.
ASCL1	achaete-scute family bHLH transcription factor 1	transcription factor, accessing closed chromatin to allow other factors to bind and activate neural pathways. Plays a role at early stages of development of specific neural lineages in most regions of the CNS, and of several lineages in the PNS. Essential for the generation of olfactory and autonomic neurons
TP53	tumor protein p53	dysregulated p53 activity may contribute to various peripheral and brain alterations during the earliest stages of AD.
NEUROG2	neurogenin 2	play an important role in neurogenesis. induces diverse neuron types from human pluripotency
FOXA2	forkhead box A2	regulate both the birth of dopamine neurons and their spontaneous death, two major goals of regenerative medicine.
LMX1A	LIM homeobox transcription factor 1 alpha	Lmx1a and Lmx1b function cooperatively to regulate proliferation, specification, and differentiation of mDA progenitors. Midbrain dopaminergic (mDA) neurons have diverse roles in regulating motor and cognitive functions
NEUROD1	neuronal differentiation 1	essential for the survival and maturation of adult-born neurons
ISL2	ISL LIM homeobox 2	multiple aspects of motor neuron development, including motor neuron cell body localization, motor column formation, and axon growth. and is required for survival of cranial ganglia neurons.
NEUROG1	neurogenin 1	*Neurog1* and *Neurog2* coordinately regulate development of the olfactory system
PITX3	paired like homeodomain 3	is important for the differentiation and maintenance of midbrain DA neurons during development
GATA2	GATA binding protein 2	necessary and sufficient to activate the transcription factors Lmx1b and Pet1, and to induce 5-HT neurons
GATA3	GATA binding protein 3	associated with early motor neuron and interneuron precursors
POU3F2	POU class 3 homeobox 2	Sufficient to Convert Astrocytes into Neural Progenitors and Neurons
LHX3	LIM homeobox 3	generation of locomotion, motor neurons and V2 interneurons.
PHOX2B	paired like homeobox 2B	regulates neuronal maturation in the brain stem nuclei associated with cardiorespiratory function and in the autonomic sympathetic and enteric nervous system
CAMK2D	Calcium/calmodulin-dependent protein kinase type II delta chain	Calcium signaling is crucial for several aspects of plasticity at glutamatergic synapses.
KL	Klotho	tole in human brain aging.
TERT	telomerase	preserves neuron survival and cognition

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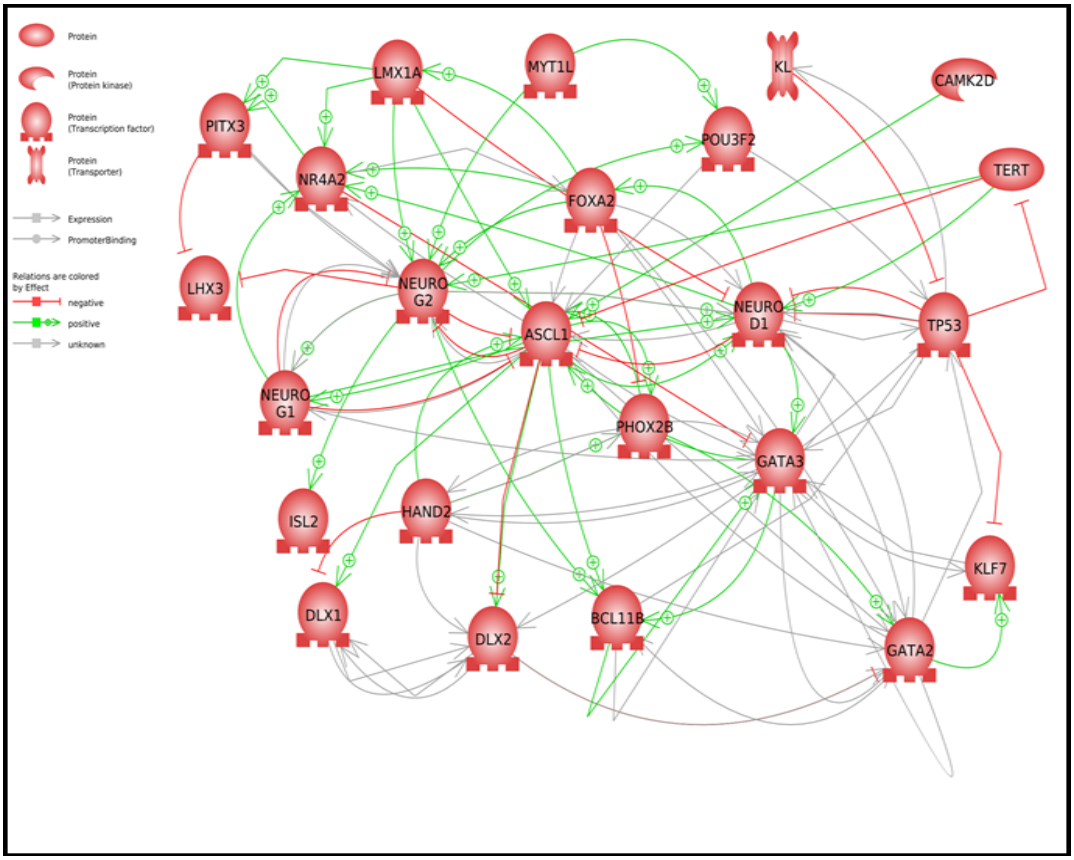

Figure 2: Pathway Studio analysis network of neuronal TFs

10,000 rpm for 15 min. Finally, we transferred the supernatant to a fresh tube and determined the protein concentration using the Bradford method, where 25 µg of protein was mixed with 1x sample loading dye containing SDS and loaded on a gel. Under denaturing conditions, we separated the proteins using Tris-glycine running buffer.

Western Blotting

We transferred the proteins to methanol-activated polyvinyl difluoride membranes (Invitrogen, USA) using a Turbo transblot system (Bio-Rad, USA). We blocked the membranes with 5% BSA for 1 h and incubated them with the appropriate primary antibody for each expressed transcription study overnight at 4°C followed by a species-specific secondary antibody for 1 h at room temperature. We rinsed the blots and incubated them with enhanced chemiluminescence (ECL) substrate (Merck, USA) for 1 min in the dark and captured the images at suitable exposure settings using a ChemiDoc XRS system (Bio-Rad, USA).

Results

Pathway Studio analysis of neuronal TFs and gene expression

Table 2 depicts a list of TFs known to play a crucial role in neurogenesis and neuronal differentiation, and Table 3 shows a list of TFs involved in the development of brain tissue and neuronal connections [21-29].

To further understand the direct interactions among neuronal TFs, we analyzed gene networks using Pathway Studio software. Figure 2 includes protein–protein interaction maps and shows a closed feedback loop network. A complete list of cellular processes regulated by expressed genes is available in the Supplementary Material. The top five gene processes regulated by the neuronal gene set ($p < e^{-15}$) were neuron development, neuron differentiation, nervous system development, stem cell differentiation, and nerve cell differentiation (Table 4).

qRT–PCR analysis of TF expression in fibroblasts treated with Metadichol®

We performed qRT–PCR of all genes listed in Table 3 in fibroblasts treated with Metadichol®. All TFs in Table 3 were detectable, with varying changes in expression. Most TFs showed multi fold increases after treatment with Metadichol®. Down regulation was seen for protein 53 (*TP53*), neuronal differentiation 1 (*NEUROD1*), LIM homeobox 3 (*LHX3*), and myelin transcription factor 1 (*MYT1L*). Only marginal increases were observed for nuclear receptor subfamily 4, group A (*NR4A2*), LIM homeobox transcription factor 1 alpha (*LMXA1*), GATA binding protein 2 (*GATA2*), and distal-less homeobox 1 (*DXL1*).

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Table 4: Top five cell processes regulated by the neuronal gene set identified by Pathway Studio analysis (Elsevier)

Gene Set Seed	Cell processes regulated	Total # of Neighbors	Overlap	Percent Overlap	Overlapping Entities	p value
neuron development	Protein regulators of neuron development	484	21	4	NEUROG,FOXA,LMX1A;NEUROD1;ISL,NEUROG1;TERT;PITX3;GATA,GATA3;POU3F,LHX3;PHOX2B;KLF7;NR4A,HAND,DLX,MYT1L;BCL11B;ASCL1;TP53	1.23E-28
neuron differentiation	Protein regulators of neuron differentiation	380	18	4	NEUROG,LMX1A;NEUROD1;ISL,NEUROG1;PITX3;GATA,GATA3;POU3F,LHX3;PHOX2B;KLF7;NR4A,DLX1;HAND,BCL11B;ASCL1;TP53	2.74E-24
nervous system development	Protein regulators of nervous system development	1674	21	1	NEUROG,FOXA,LMX1A;NEUROD1;NEUROG1;TERT;GATA,GATA3;POU3F,KL;LHX3;PHOX2B;KLF7;NR4A,DLX1;HAND,DLX,MYT1L;BCL11B;ASCL1;TP53	2.71E-17
stem cell differentiation	Protein regulators of stem cell differentiation	2141	22	1	NEUROG,FOXA,LMX1A;NEUROD1;NEUROG1;TERT;PITX3;GATA,GATA3;POU3F,KL;LHX3;PHOX2B;KLF7;NR4A,DLX1;HAND,DLX,MYT1L;BCL11B;ASCL1;TP53	1.02E-16
nerve cell differentiation	Protein regulators of nerve cell differentiation	2182	22	1	NEUROG,FOXA,LMX1A;NEUROD1;ISL,NEUROG1;TERT;PITX3;GATA,GATA3;POU3F,LHX3;PHOX2B;KLF7;NR4A,DLX1;HAND,DLX,MYT1L;BCL11B;ASCL1;TP53	1.55E-16

Table 5: q-RT–PCR raw data and TF fold changes

Sample	Actin	PHOX2 B	Delta ct	Delta ct	Fold change 2^DDct	Sample	Actin	KLF 7	Delta ct	Delta ct	Fold change 2^DDct
Control	20.08	31.688	11.609	0	1	Control	20.08	28.654	8.58	0	1
1 pg	19.62	27.833	8.209	-3.4	10.559	1 pg	19.62	27.86	8.24	-0.339	1.265
100 pg	19.42	29.659	10.236	-1.373	2.59	100 pg	19.42	26.694	7.27	-1.304	2.468
1 ng	19.19	30.977	11.789	0.18	0.883	1 ng	19.19	25.165	5.98	-2.597	6.052
100 ng	19.23	28.835	9.601	-2.008	4.023	100 ng	19.23	23.921	4.69	-3.888	14.81
Sample	Actin	FOXA2	Delta ct	Delta ct	Fold change 2^DDct	Sample	Actin	NEUROG2	Delta ct	Delta ct	Fold change 2^DDct
Control	20.08	20.42	0.34	0	1	Control	20.08	27.59	7.52	0	1
1 pg	19.62	19.32	-0.3	-0.645	1.56	1 pg	19.62	25.64	6.01	-1.5	2.83
100 pg	19.42	18.02	-1.4	-1.744	3.35	100 pg	19.42	25.49	6.07	-1.45	2.73
1 ng	19.19	16.9	-2.29	-2.629	6.19	1 ng	19.19	25.44	6.25	-1.26	2.4
100 ng	19.23	18.48	-0.75	-1.096	2.14	100 ng	19.23	25.35	6.12	-1.4	2.63
Sample	Actin	ISL2	Delta ct	Delta ct	Fold change 2^DDct	Sample	Actin	DXL1	Delta ct	Delta ct	Fold change 2^DDct
Control	20.08	20.19	0.11	0	1	Control	20.08	23.28	3.2	0	1
1 pg	19.62	19.27	-0.35	-0.46	1.38	1 pg	19.62	22.21	2.59	-0.62	1.53
100 pg	19.42	18.21	-1.22	-1.33	2.51	100 pg	19.42	22.16	2.74	-0.46	1.38
1 ng	19.19	15.83	-3.36	-3.47	11.08	1 ng	19.19	22.9	3.71	0.51	0.7
100 ng	19.23	19.13	-0.11	-0.22	1.16	100 ng	19.23	22.31	3.08	-0.13	1.09
Sample	Actin	DXL2	Delta ct	Delta ct	Fold change 2^DDct	Sample	Actin	MYT1L	Delta ct	Delta ct	Fold change 2^DDct
Control	20.08	32.14	12.06	0	1	Control	20.08	15.78	-4.29	0	1

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1 pg	19.62	29.74	10.12	-1.94	3.85	1 pg	19.62	15.4	-4.22	0.07	0.95
100 pg	19.42	28.25	8.83	-3.23	9.38	100 pg	19.42	15.52	-3.9	0.39	0.76
1 ng	19.19	27.99	8.81	-3.25	9.54	1 ng	19.19	15.64	-3.55	0.75	0.6
100 ng	19.23	28.62	9.38	-2.68	6.41	100 ng	19.23	15.59	-3.64	0.65	0.64
Sample	Actin	GATA2	Delta ct	Delta ct	Fold change 2^DDct	Sample	Actin	GATA3	Delta ct	Delta Delta ct	Fold change 2^DDct
Control	20.08	20.43	0.35	0	1	Control	20.08	21.03	0.95	0	1
1 pg	19.62	19.89	0.26	-0.09	1.06	1 pg	19.62	19.19	-0.43	-1.38	2.6
100 pg	19.42	19.86	0.44	0.09	0.94	100 pg	19.42	19.57	0.15	-0.8	1.74
1 ng	19.19	20.42	1.24	0.89	0.54	1 ng	19.19	18.77	-0.42	-1.37	2.58
100 ng	19.23	18.53	-0.7	-1.05	2.07	100 ng	19.23	17.7	-1.54	-2.49	5.6
Sample											
Control	Actin	NR4A2	Delta ct	Delta Delta ct	Fold change 2^DDct	Sample	Actin	ASCL1	Delta ct	Delta Delta ct	Fold change 2^DDct
1 pg	20.08	10.84	-9.23	0	1	Control	20.08	20.23	0.15	0	1
100 pg	19.62	9.58	-10.04	-0.81	1.75	1 pg	19.62	17.96	-1.66	-1.82	3.52
1 ng	19.42	9.96	-9.46	-0.23	1.17	100 pg	19.42	18.5	-0.92	-1.08	2.11
100 ng	19.19	10.59	-8.6	0.64	0.64	1 ng	19.19	14.73	-4.46	-4.61	24.42
	19.23	10.23	-9	0.23	0.85	100 ng	19.23	18.93	-0.3	-0.45	1.37
Sample											
Control	Actin	TP53	Delta ct	Delta Delta ct	Fold change 2^DDct	Sample	Actin	BCL11B	Delta ct	Delta Delta ct	Fold change 2^DDct
1 pg	20.08	18.08	-2	0	1	Control	20.08	21.129	1.05	0	1
100 pg	19.62	18.3	-1.32	0.68	0.62	1 pg	19.62	20.158	0.53	-0.52	1.43
1 ng	19.42	17.9	-1.29	0.71	0.61	100 pg	19.42	18.528	-0.89	-1.94	3.85
100 ng	19.19	16.57	-2.61	-0.61	1.53	1 ng	19.19	17.951	-1.24	-2.29	4.88
	19.23	18.06	-1.18	0.83	0.56	100 ng	19.23	18.922	-0.31	-1.36	2.57
Sample											
Control	Actin	LHX3	Delta ct	Delta Delta ct	Fold change 2^DDct	Sample	Actin	LMXA1	Delta ct	Delta Delta ct	Fold change 2^DDct
1 pg	20.08	25.39	5.31	0	1	Control	20.08	28.83	8.75	0	1
100 pg	19.62	26.64	7.02	1.71	0.31	1 pg	19.62	28.21	8.59	-0.16	1.12
1 ng	19.42	26	6.58	1.27	0.41	100 pg	19.42	28.21	8.79	0.04	0.97
100 ng	19.19	25.59	6.41	1.1	0.47	1 ng	19.19	27.12	7.93	-0.82	1.76
	19.23	25.97	6.74	1.43	0.37	100 ng	19.23	27.9	8.66	-0.09	1.07
Sample											
Control	Actin	NEUROD1	Delta ct	Delta Delta ct	Fold change 2^DDct	Sample	Actin	HAND2	Delta ct	Delta Delta ct	Fold change 2^DDct
1 pg	20.08	18.71	-1.37	0	1	Control	20.08	27.76	7.68	0	1
100 pg	19.62	18.69	-0.93	0.43	0.74	1 pg	19.62	26.53	6.91	-0.77	1.71
1 ng	19.42	18.5	-0.92	0.45	0.73	100 pg	19.42	25.96	6.54	-1.14	2.2
100 ng	19.19	19.14	-0.04	1.33	0.4	1 ng	19.19	25.46	6.27	-1.41	2.65
	19.23	18.66	-0.57	0.8	0.58	100 ng	19.23	26.06	6.83	-0.85	1.81
Sample											
Control	Actin	POU3F2	Delta ct	Delta Delta ct	Fold change 2^DDct	Sample	Actin	PITX3	Delta ct	Delta Delta ct	Fold change 2^DDct
1 pg	20.08	25.8	5.72	0	1	Control	20.08	30.5	10.42	0	1
100 pg	19.62	25.88	5.8	0.08	0.95	1 pg	19.62	29.93	9.85	-0.57	1.49
1 ng	19.42	25.03	4.95	-0.77	1.7	100 pg	19.42	28.68	8.6	-1.82	3.52

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100 ng	19.19	23.05	2.97	-2.75	6.73	1 ng	19.19	27.83	7.76	-2.67	6.34
	19.23	24.5	4.42	-1.3	2.47	100 ng	19.23	28.12	8.04	-2.38	5.2
Sample											
Control	Actin	NEUROG1	Delta ct	Delta Delta ct	Fold change 2^DDct	Sample	Actin	CAMK2D	Delta ct	Delta Delta ct	Fold change 2^DDct
1 pg	20.08	30.835	10.76	0	1	Control	20.08	29.28	9.2	0	1
100 pg	19.62	28.905	9.28	-1.48	2.78	1 pg	19.62	28.91	9.28	0.08	0.95
1 ng	19.42	27.475	8.05	-2.7	6.52	100 pg	19.42	27.13	7.7	-1.5	2.83
100 ng	19.19	25.925	6.74	-4.02	16.21	1 ng	19.19	25.41	6.22	-2.98	7.88
	19.23	28.525	9.29	-1.47	2.76	100 ng	19.23	28.16	8.92	-0.28	1.21
Sample											
Control	Actin	Klotho	Delta ct	Delta Delta ct	Fold change 2^DDct	Sample	Actin	Telomerase	Delta ct	Delta Delta ct	Fold change 2^DDct
1 pg	20.08	26.61	5.76	0	1	Control	20.08	25.596	5.517	0	1
100 pg	19.62	24.8	4.26	-1.5	2.82	1 pg	19.62	23.26	3.636	-1.881	3.683
1 ng	19.42	23.76	2.42	-3.34	10.13	100 pg	19.42	23.013	3.59	-1.927	3.802
100 ng	19.19	28.3	7.68	1.92	0.26	1 ng	19.19	22.199	3.012	-2.505	5.678
	19.23	28.9	8.15	2.39	0.19	100 ng	19.23	22.748	3.514	-2.003	4.008

Discussion

Downregulation of ***TP53*** expression has been shown to increase the efficiency of iPSC production [30]; inactivation of the p53 pathway is a prerequisite for successful reprogramming of somatic cells into neuronal or other cell types [31]. **Klotho** gene (*KL*) expression increased 10-fold after treatment with 1 ng Metadichol®. Klotho, a type 1 transmembrane protein, has been shown to inhibit TP53 and regulate cellular senescence by repressing the p53/p21 pathway [32]. Thus, klotho prevents aging in primary human fibroblasts [33] and is neuroprotective. Maintaining Klotho levels by stimulating endogenous Klotho production may [34] be a potential therapeutic target to slow the incidence of age-related diseases.

Some TFs whose expression increased or decreased after treatment with Metadichol® play critical roles in neuronal processes and disease. For instance, ***NEUROD1*** is expressed in aggressive neuroendocrine tumors, malignant melanoma, and prostate cancer cell lines [35]. Moreover, it is a therapeutic target for aggressive neuroendocrine cancers expressing NEUROD1 [36]. Moreover, paired-like homeobox 2b (***PHOX2b***) expression increased over 10-fold after treatment with 100 pg (pico gram). Metadichol®; PHOX2b has been shown to suppress neuroblastoma progression [37,38,39]. Kinesin family member 7 (***KIF7***) exhibited a 14-fold increase in expression after treatment with 100 ng Metadichol®. KIF7 maintains normal brain function; recent work [40] suggests that it plays a crucial role in the pathogenesis of stroke, providing vascular and neuronal protection in cases with an ischemic brain. BHLH transcription factor 1 (***ASCL1***) exhibited a 24-fold increase after 1 ng of treatment. ASCL1 levels are low in glioblastoma (GBM) tumors [41]; thus, increasing ASCL1 expression plays a role in controlling GBM. [42] ASCL1 induces the expression of TFs responsible for the differentiation of dopaminergic neurons, such as POU class 3 homeobox 2 (***POU3F2***) and forkhead box A2 (***FOXA2),*** both of which exhibited a sixfold increase in expression after treatment with 1 ng Metadichol®. Moreover, **FOXA2** plays a crucial role in the birth and death of dopaminergic neurons and in the development of PD [43], as the gradual loss of dopaminergic neurons is a hallmark of this disease. The pituitary homeobox 3 (***PITX3***) gene showed a sixfold increase in expression with 1 ng of Metadichol® treatment and has a similar role to FOXA2 in the differentiation and maintenance of midbrain neurons [44]. Calcium/calmodulin-dependent protein kinase 2 (***CAMK2D***) exhibited a nearly eightfold increase in expression after 1 ng of Metadichol® treatment. As CAMK2D can phosphorylate itself, its activity is independent of Ca^{2+}/calmodulin and thus helpful for the formation of long-term memory [45]. ***GATA2*** (twofold increase at 100 ng) increases the yield of serotonergic neurons [46]. ***POU3F2*** expression increased sixfold after 1 ng of Metadichol® treatment; *POU3F2* has been shown to regulate the gene coexpression network involved in schizophrenia and bipolar disorders [47]. FOXA2 [48] activates serotonergic neural differentiation pathways, leading to a 10-fold increase in serotonin production. ***GATA3*** exhibited a sixfold increase with 100 ng of Metadichol® treatment; it enhances the neurogenic potential [49] of primary human astrocytes but cannot induce neurogenesis on its own. The expression of neurogenin 2 (***NEUROG2***) increased nearly threefold after treatment with 100 ng Metadichol®. Its absence halts neurogenesis [50]. BAF chromatin remodeling complex subunit (***BCL11b***) expression increased fivefold after treatment with Metadichol®. BCL11b is a TF with a

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crucial role in fetal development, as it is necessary for the differentiation and development of neuronal subtypes in the central nervous system [51]. Heart and neural crest derivative-expressed protein 2 (***HAND2)*** exhibited a twofold increase in expression with 1 ng of Metadichol® treatment. HAND2 is involved in neurogenesis as well as in the expression of cell-specific markers in the enteric nervous system [52]. Neurogenin 1 **(*NEUROG1*)** is a TF important for sensory neuron development; it affects hair cells, having a minor impact on cochlear nuclei development [53]. Its expression increased 16-fold after treatment with 1 ng Metadichol®. ISL LIM Homeobox 2 (***ISL2***) exhibited an 11-fold increase after treatment with 1 ng Metadichol®. ISL2 is mainly expressed in primary sensory and motor neurons and is essential for the acquisition of motor neuron identity [54]. Telomerase (***TERT***) expression increased sixfold after treatment with 1 ng Metadichol®. Telomerase promotes neurogenesis [55] during neural injury and repair in patients with various diseases affecting the nervous system. Telomerase and Klotho expression in the brain can ameliorate brain aging and neurodegenerative diseases such as AD and PD [56,57,58].

Vitamin C and oxidative stress in fibroblasts treated with Metadichol®

A recent review points out the importance of ascorbate (vitamin C) and its vital role in the brain [59]. Ascorbate (vitamin C) accumulates at the highest level in brain tissues, at concentrations between 2–10 μM, while blood levels are only 40–60 mM [60]. Ascorbate is a neuromodulator of glutamine, dopamine, cholinergic (61) and GABA. Ascorbate is a cofactor in catecholamine and collagen production and is involved in the regulation of hypoxia-induced factor-1α [62.63]. Ascorbate has been shown to spare/recycle α-tocopherol in lipid bilayers [64] and erythrocytes [65].

Our previous work showed that oral administration of Metadichol® increases ascorbate levels in humans [66,67]. Dehydroascorbic acid (DHA) is the oxidized form of ascorbate and can be transported via glucose transporters of the GLUT family, such as GLUT 4. Metadichol® increases GLUT4 expression [68], suggesting that Metadichol® can enhance the ability of cells to import DHA, and once inside, it is rapidly reduced to ascorbate.

Oxidative stress in the brain is a leading cause of neurodegenerative diseases [69]. Neurons are greatly affected by ascorbate deficiency, possibly due to their 10-fold higher oxidative metabolism than other cell types [70]. Ascorbate is important for neuronal maturation and function and for protecting the brain against oxidative stress [71]. AD patients have lower ascorbate levels in plasma [72] and CSF [73] despite adequate nutritional intake. Ascorbate use was found to reduce disease incidence [74,75] and disease-related oxidative stress markers [76]. Inclusion of ascorbate in cell media could efficiently enhance the generation of mouse embryonic stem cells and iPSCs from mouse or human fibroblasts [77] compared to other antioxidants. Ascorbate might act by promoting the expression of particular genes. For example, ascorbate can facilitate cell fate reprogramming by increasing histone demethylation [78]. Moreover, ascorbate has been shown to increase the embryonic stem cell population in humans, leading to DNA demethylation at genomic loci known to undergo widespread methylation loss during reprogramming of somatic cells into iPSCs [79,80].

The findings provided in this study demonstrate that the administration of Metadichol leads to the activation of several genes associated with neuronal function. It is not possible for a single small molecule to activate all genes associated with neuronal function. There is a higher probability that Metadichol activates up field genes in the pathway, which subsequently triggers the activation of a cascade of downstream genes. Our recent study has demonstrated that Metadichol exhibits the expression of all nuclear receptors in both stem cells and fibroblast cells [81]. Nuclear receptors exhibit selective expression among neuronal populations [82].

Retinoic acid receptors (RARs) and Retinoid X receptors (RXRs) are two types of nuclear receptors that play crucial roles in mediating the effects of retinoic acid, a derivative of vitamin A. The activation of these nuclear receptors occurs by the binding of retinoic acid, which is a product of vitamin A. The involvement of retinoic acid signaling in neuronal differentiation and maturation has been demonstrated to be significant [83]. The RXRA protein plays a crucial role in the signaling pathway of retinoic acid (RA), governing the production of genes and the functioning of neurons across different brain areas. The RXRA gene [84] plays a crucial role in the proper development and functioning of the nervous system, as well as in the manifestation of behavioral abnormalities. The involvement of retinoic acid in the process of neural development is somewhat facilitated by its ability to regulate neurogenic transcription factors, such as NGN3 and PAX6 these factors are fundamental in the differentiation and development of various neuronal cell types.

Orphan nuclear receptors, namely TLX (NR2E1) and Nurr1 (NR4A2), have demonstrated their involvement in the processes of neural stem cell self-renewal and differentiation [85,86]. Although the direct regulation of NGN3 and PAX6 by these receptors is not established, their involvement in wider networks of neuronal growth and differentiation has been implicated.

Thyroid B Nuclear receptor (THRB) plays a significant role in the modulation of neuronal excitability. The activation of THRB has been demonstrated to enhance the transcription of genes implicated in the synthesis of neurotransmitters [87,88].

The nuclear receptor GR is involved in the response

to stress. GR activation has been shown to decrease the expression of genes that are involved in learning and memory [89,90].

Our results demonstrate that Metadichol® enhanced the expression of many TFs associated with neuronal regulation, as identified by Pathway Studio software Interactions between neuronal factors affect processes such as neuron differentiation, maintenance of neurogenesis pluripotency, cellular senescence, and stem cell development (Figure 2). The identified genes have more interactions among themselves than expected for a random set of genes of the same size and degree distribution drawn from the genome. Such enrichment indicates that these genes share a significant biological connection through their involvement in neuronal development.

Our work was triggered by post marketing field reports from customers using Metadichol® over the last 5 years. This result suggested that symptoms in neurological syndromes such as attention-deficit hyperactivity disorder (ADHD), bipolar disorder, and autism spectrum disorders improved. Our results suggest that future work should focus on directly testing the effects of Metadichol® on patients with neurological diseases, given that it is nontoxic and has no known side effects [91,92,93]. Metadichol®, as we have shown, acts on multiple targets or disease pathways by activating nuclear receptors and and lead to a tightly bound gene network that can bring about changes in neurodegenerative diseases where such a need exists.

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Supplementary Files Enclosed.

1) Western Blots
2) Raw Q-Rt-PCR data
3) Biological processes regulated by the expressed gene sets.

Supplementary Files link

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