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ORIGINAL ARTICLE

A pharmacogenomic profile of human neural progenitors undergoing differentiation in the presence of the traditional Chinese medicine NeuroAiD

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NeuroAiD, a traditional Chinese medicine widely used to treat stroke patients in China, was recently demonstrated in rodent models and in clinical trials to possess neuroregenerative and neuroprotective properties. In order to understand the mechanisms employed by NeuroAiD to bring about its neuroproliferative and neuroprotective effects, we investigated the impact of MLC901, a reformulated version of MLC601, on human neural progenitors undergoing neural differentiation at the molecular level by performing three independent microarray experiments. Functional annotations of the genes regulated by MLC901 that were associated with neurogenesis were found to be enriched. We also identified potential targets (*FGF19*, *GALR2*, *MMP10*, *FGF3* and *TDO2*) of MLC901 that could promote neurogenesis and neuroprotection in the human brain. This work highlighted some interesting targets and offered some insights into the possible mechanism of action of MLC901. The discovery could also provide a platform to the development of future therapeutic targets.

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INTRODUCTION

NeuroAiD, a Traditional Chinese medicine that is registered with the Sino Food and Drug Administration, is widely used to treat stroke patients in China.[1] NeuroAiD MLC601, which contains nine herbal components plus five animal components, and MLC901, which is formulated with only the herbal components, have demonstrated efficacy in rodent models of ischemic stroke.[2] Clinical trials with MLC601 have shown improvements in functional outcomes of subjects presenting with cerebral infarction.[3–6] Results from *in vitro* models using cultured cortical neurons show that MLC601/901 prevents neuronal cell death induced by the excitotoxic challenge of glutamate.[7] Other *in vitro* studies have shown that NeuroAiD induces neurogenesis, neuroproliferation and neurite outgrowth.[2,7,8] Given the efficacy of NeuroAiD we sought to learn more about its mechanism of action on human neurons. We hypothesized that examination of the transcriptional response in human neurons to NeuroAiD would lead to clues as to the cellular and molecular basis of its actions.

In order to investigate the effect of NeuroAiD on neurogenesis, we investigated the impact of MLC901 on human neural progenitor cells (NPC) undergoing neural differentiation. To generate consistent batches of NPC and neurons of human origin, an *in vitro* differentiation methodology was adopted. Starting with human embryonic stem cells (hESC), directed differentiation generated NPC, which were subsequently directed to generate neurons giving us sufficient numbers of cells to perform detailed transcriptome profiling using DNA microarrays. Analysis of the gene expression changes revealed genes of functional pathways regulated by MLC901. This work highlights some interesting targets and offers insights into the possible mechanism of action of MLC901.

MATERIALS AND METHODS

Neural induction

H9 hESC (WiCell, Madison, WI, USA) were cultured on Matrigel (Becton Dickinson, Bedford, MA, USA) in mTeSR1 (Stem Cell Technologies, Vancouver, British Columbia, Canada) prior to neural induction. Monolayer neural induction of the hESC was carried out as described in Li *et al*.[9] The neural induction media contained 10 ng ml^{-1} hLIF (Millipore, Temecula, CA, USA), 3 μM CHIR99021 (Tocris, Bristol, UK), 2 μM SB431542 (Tocris) and 0.1 μM γ-secretase inhibitor XXI, compound E (Millipore) in N2B27 media (Dulbecco's Modified Eagle's Medium/F12:Neurobasal (1:1), N2, B27 without vitamin A, 1% glutamax, 5 μg ml^{-1} bovine serum albumin). The neural induction media was changed daily during the neural induction period. At the end of day 7, the NPC were passaged as single cells at 1:6 with Accutase (Millipore) and maintained in neural progenitor expansion media (NEM) consisting of 3 μM CHIR99021, 2 μM SB431542, 20 ng ml^{-1} epidermal growth factor and 20 ng ml^{-1} basic fibroblast growth factor (FGF) in N2B27 media. These NPC were frozen as single cells in 10% dimethyl sulfoxide (DMSO)/NEM at passage 3 and kept at −150 °C as stocks for the neural differentiation experiments.

Neural differentiation

H9-derived NPC were differentiated as described in Li *et al*.[9] with neural differentiation medium (NDM) comprising Dulbecco's Modified Eagle's Medium/F12, N2B27, 300 ng ml^{-1} cAMP (Sigma-Aldrich, Buchs, Switzerland), 0.2 mM vitamin C, 3 μM CHIR99021, 2 μM SB431542, 10 ng ml^{-1} brain-derived neurotrophic factor (BDNF) and 10 ng ml^{-1} glial-derived neurotrophic factor (GDNF). The basal NDM was essentially NDM without BDNF and GDNF and in place of B27 was B27 without antioxidants. The NPC were plated as single cells at a density of 5 00 000 cells per well of poly-L-lysine (Sigma P4707) and laminin (Sigma L2020; 10 μg ml^{-1})-coated six-well plates in NEM. On day 3, the NEM was replaced with NDM or basal NDM with or without MLC901. For gene expression profiling studies, duplicates were set up for each time points,

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the entire experiment was independently replicated on another day and all data collected were included in subsequent analyses.

Drug treatment preparation

MLC901 was provided by Moleac PTE (Singapore) in powder form. Refer to Heurteaux *et al.* [7] for the drug composition. The concentration used for the *in vitro* experiments was 1 μg ml^{-1}. A stock of 10 mg ml^{-1} MLC901 was made by incubating the powdered MLC901 in Dulbecco's Modified Eagle's Medium/F12 at 37 °C for an hour and filtered with a 0.22- μm filter before diluting further with Dulbecco's Modified Eagle's Medium/F12 to 1 mg ml^{-1} treatment stock, which was added to the NDM or basal NDM at a concentration of 1 μg ml^{-1}.

Immunostaining

NPC or neuronal cells were fixed with 4% paraformaldehyde for 15 min on ice, washed twice with phosphate-buffered saline followed by phosphate-buffered saline + 0.1% Tween (PBT) for 5 min each time. Thereafter, the cells were blocked with PBT containing 10% fetal bovine serum for 30 min at room temperature after which they were incubated overnight at 4 °C in blocking solution containing the desired antibodies in the following dilutions: mouse anti-Oct4 (Santa Cruz, Dallas, TX, USA; sc-5279; 1:100), rabbit anti-Pax6 (Covance, Princeton, NJ, USA; PRB-278P; 1:500), mouse anti-Nestin (Millipore; MAB5326; 1:1000), goat anti-doublecortin (Santa Cruz; sc-8066; 1:400), mouse anti-Neuronal nuclei (NeuN) (Millipore; MAB377; 1:100) and mouse anti-microtubule-associated protein 2 (Stem Cell Technologies; 01410; 1:200). Cells were washed thrice with PBT the next day and stained with Life Technologies secondary antibody (AlexaFluor 488 (A11029)/594 (A11032) goat anti-mouse IgG, AlexaFluor 488 (A11034)/594 (A11037) goat anti-rabbit IgG and AlexaFluor 594 (A11058) donkey anti-goat IgG] diluted in PBT+10% normal goat serum for an hour at room temperature in the dark. The cells were washed 3× with PBT and stained with 4′,6-diamidino-2-phenylindole (DAPI) (10 μg ml^{-1}) before imaging with the Zeiss Axio Imager.Z1 microscope.

Image analysis

An average of four or five images were taken for each antibody at three different time points (days 3, 7 and 14). The mean fluorescence intensities of the images were measured with a cell image analysis software, CellProfiler.[10] The average mean fluorescence intensity values were plotted against the time points for each neuronal marker.

RNA extraction

Extraction of RNA from NPC was performed by combining the TRIzol reagent (Invitrogen) and RNeasy micro kit (Qiagen, Valencia, CA, USA) protocols. Each well of cells lysed in 1 ml of TRIzol was transferred to a MaXtract high density tube (Qiagen), mixed with 0.2 ml chloroform (Sigma; C2432) and vortexed for 15 s before incubation at room temperature for 3 min. Phase separation was accomplished by centrifugation at 12 000 *g* for 15 min at 4 °C. The RNA was precipitated with an equal volume of 70% ethanol, mixed and applied to the RNeasy MinElute spin columns.

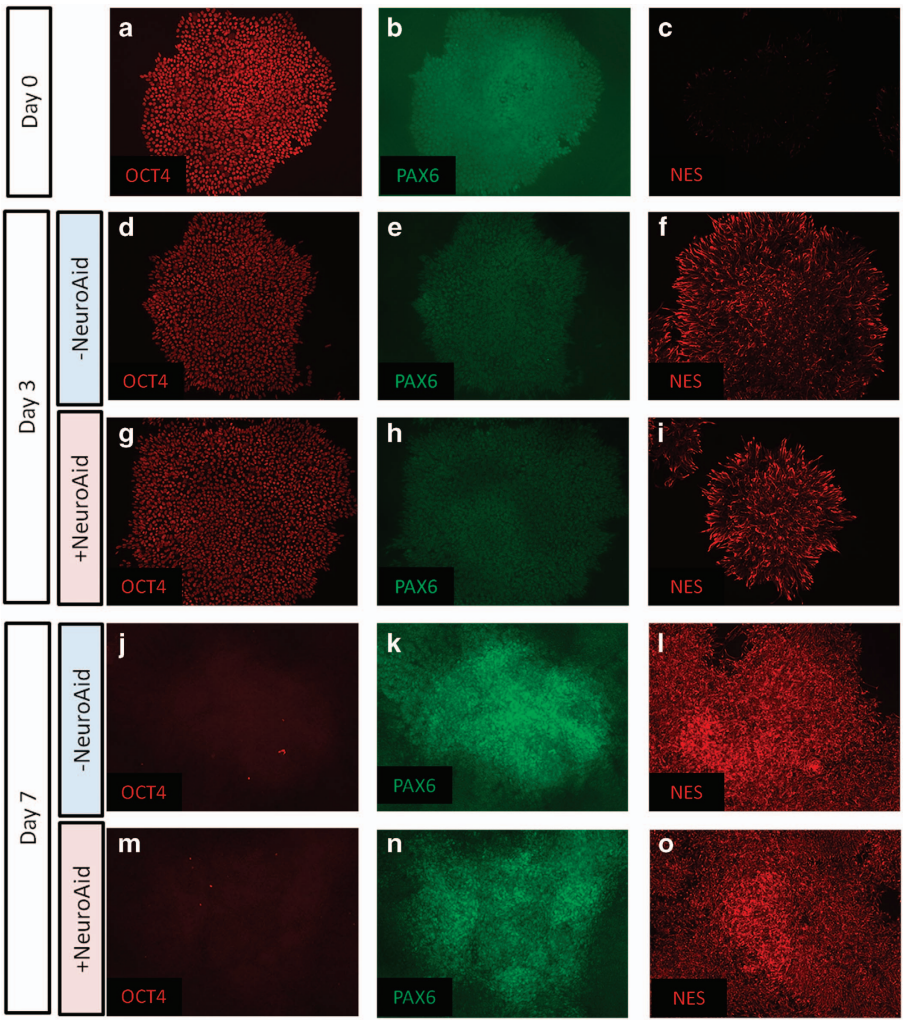

Figure 1. Neural induction of H9 hESC to NPC over 7 days in the absence and presence of NeuroAiD. The decreasing expression of stem cell marker, Oct4, concomitant with the increasing expressions of neural progenitor markers, Pax6 and Nestin (NES), display the successful neural induction. NeuroAiD has no observable impact on the induction process (**d**–**f**, **j**–l versus **g**–**i**, **m**–**o**). Magnification: 100×. hESC, human embryonic stem cells; NPC, neural progenitor cells.

Thereafter, the RNA extraction proceeded according to the manufacturer's instructions.

RNA amplification

The RNA amplification was performed using the Illumina TotalPrep RNA Amplification kit (Ambion, Carlsbad, CA, USA; #IL1791) according to the manufacturer's protocol. Five hundred nanograms of extracted and purified total RNA from each sample was used as the starting material for the amplification to produce sufficient labeled cRNA for the microarray hybridizations.

Microarray

An 18-h overnight hybridization at 58 °C was carried out on the Illumina HumanHT-12 v4 Expression BeadChips (Illumina, San Diego, CA, USA) using 750 ng of cRNA from each sample and scanned with the Illumina BeadArray Reader according to the Illumina Whole-Genome Gene Expression Direct Hybridization Assay Guide. All microarray data are publically available at the NCBI Gene Expression Omnibus (GEO accession # GSE79388).

Data analysis

The GenomeStudio V2011.1 (Illumina) software was used to extract and export the microarray data as GeneSpring format with background subtraction and no normalization. Quantile normalization and two-way analysis of variance pair-wise comparisons between the four neural differentiation time points (4, 8, 12 and 24 h) with or without MLC901 and the 0 h time point was performed with the GeneSpring GX12.5 (Agilent Technologies, Santa Clara, CA, USA) to identify transcripts that were significantly changing over time in both the treated and untreated group. The significance threshold was set at $P < 0.05$ and a fold-change ratio of more than 1.5. Transcripts that were changing exclusively in the treated groups over time were taken as differentially regulated by MLC901 while those found only in the untreated groups were ignored. Transcripts that were found significantly changing over time in both the treated and untreated groups were further scrutinized. Transcripts found changing at a particular time point in both the treated and untreated groups but at an extent difference of more than 1.5 (calculated by taking the ratio of the fold changes of the same transcript in both the treated and the untreated groups) were considered differentially expressed between the treated and the untreated groups. Together with the transcripts changing exclusively in the treated groups, these transcripts were regarded as transcripts that were differentially regulated by MLC901.

RESULTS

Testing the effect of MLC901 on human neurogenesis *in vitro*

We have developed and optimized a protocol to generate human neuronal cells from hESC.[11] This method proceeds in two phases, a neural induction phase that generates NPC and a subsequent neural specification phase that generates cells with many features of human neurons. The neural induction protocol produces cells that express progenitor markers Nestin and PAX6 after 1 week, and are negative for the stem cell pluripotency marker OCT4 (Figure 1). We tested what effect MLC901 would have on the induction of NPC from hESC. Throughout the differentiation period, cells were incubated with MLC901 (1 μg ml^{-1}) and examined at day 3 and day 7 for expression of OCT4, Nestin and PAX6. No differences were observed between the treated and untreated cells (Figure 1). Thus, we conclude that MLC901 has no impact on the induction of NPC from hESC under the directed differentiation conditions we used.

We then tested whether MLC901 had an effect on the neural specification phase of our *in vitro* differentiation protocol. NPC induced with NDM to differentiate into neuronal cells were treated with MLC901 continuously for 2 weeks. On days 3, 7 and 14 the cells were stained for expression of the neural markers microtubule-associated protein 2, doublecortin and Neuronal nuclei (Figures 2a–f). We noted a slight but consistent increase in the number of neuronal cells generated from NPC in the presence of MLC901 and this was confirmed by quantitation of the immunostaining (Figures 2g–i). Although the results did not show statistical significance at all time points, the trend suggested that there might be an effect of MLC901 on human neurogenesis under the *in vitro* conditions we

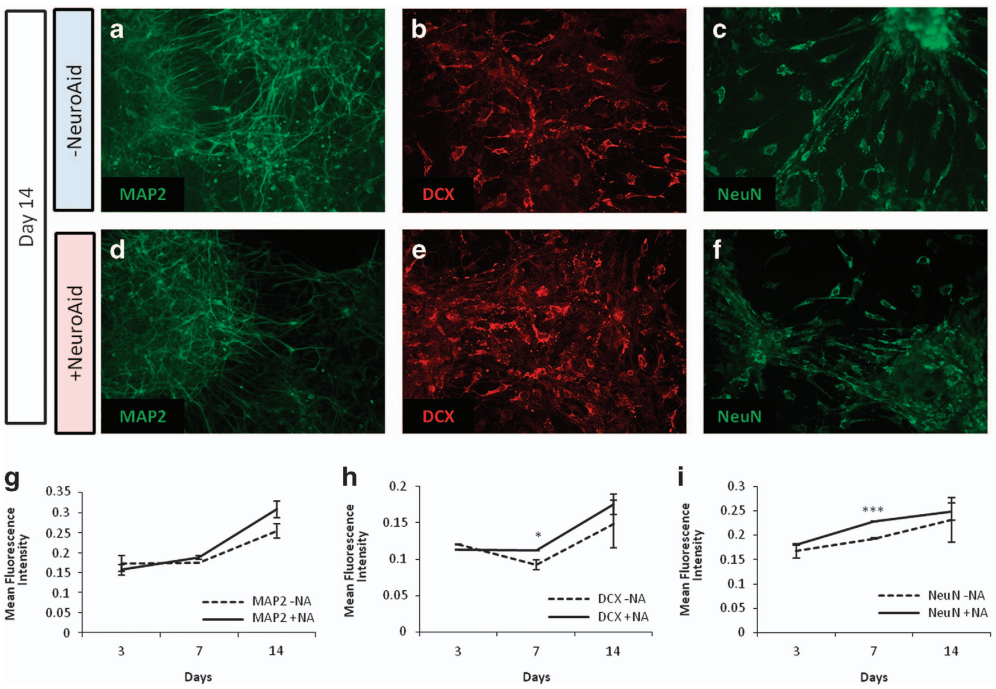

Figure 2. Neural differentiation of H9-derived neural progenitor cells (NPC) to neurons in the absence and presence of NeuroAid over 14 days. (**a–f**) Comparing the expression of neuronal markers (MAP2, DCX, NeuN) at day 14 by immunostaining. Magnification: 100×. (**g–i**) Comparing the average mean fluorescence intensity (MFI) of each neuronal marker over three time points. DCX, doublecortin; MAP2, microtubule-associated protein 2; NeuN, Neuronal nuclei.

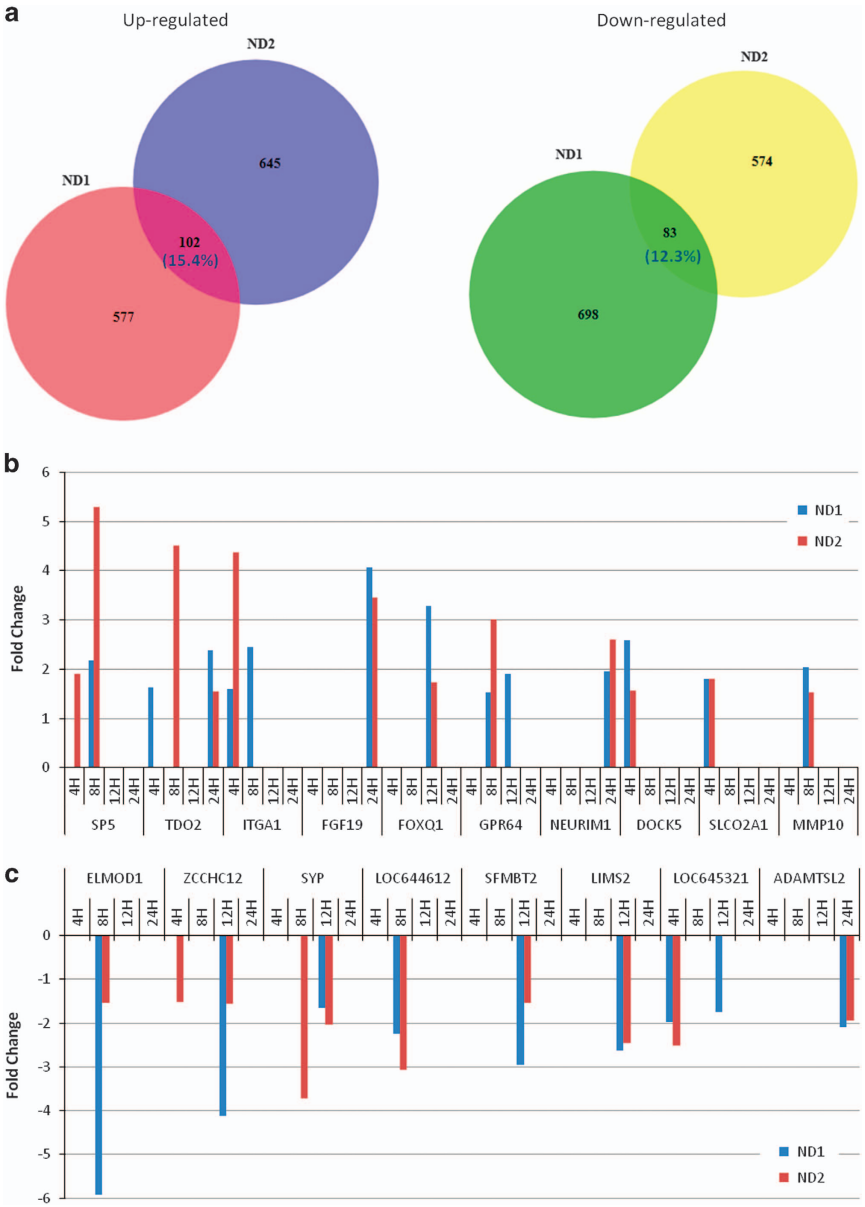

Figure 3. Genes differentially regulated by MLC901 in the first two independent microarray experiments. (**a**) A Venn diagram of the number of genes differentially regulated (fold change >1.5 and *P*-value <0.05) by MLC901. Genes commonly (**b**) upregulated (>2-fold in at least one of the experiments) and (**c**) downregulated (>2-fold in at least one of the experiments) by MLC901 at the same time points in both experiments. ND1, neural differentiation experiment 1; ND2, neural differentiation experiment 2.

tested. Given this indication of effect, we sought to examine the MLC901-treated NPC for molecular changes using gene expression profiling.

Genome-wide profiling of gene expression in NPC undergoing neural differentiation in the presence and absence of MLC901

Cultures of human neuron-like cells were produced by directed differentiation of NPC that had been generated from hESCs as described above. Gene expression profiling was performed on NPCs differentiated in the presence and absence of 1 μg ml^{-1} MLC901. To this end NPCs were cultured in NEM for 2 days before the medium was switched to NDM with or without MLC901. These differentiation-initiated neural progenitors were then harvested at early time points (0, 4, 8, 12 and 24 h after the start of differentiation) for RNA extraction, amplification and subsequent cRNA hybridization onto the Illumina HumanHT-12 Expression BeadChips (Illumina). Duplicates were set up for each time points and the entire experiment was independently replicated on another day.

There were 679 and 781 transcripts significantly up- and downregulated by MLC901, respectively, in the first set of differentiation experiment. And 747 and 657 transcripts were significantly up- and downregulated, respectively, in the second differentiation experiment (Figure 3a). There was a 15.4 and a 12.3% overlap between both sets of experiments in the upregulated and the downregulated transcripts, respectively (Figure 3a). The number of common genes that were positively regulated was 102 while 83 common genes were negatively regulated (Figure 3a). To further narrow down the significantly changing genes, only genes with a fold change of more than 2 in at least one of the independent experiment were

Table 1. Genes found commonly influenced by MLC901 in the first two independent experiments with fold change >2 in at least one experiment

Gene symbol	*Description*	*Ave FC*	*Expt*	*Time point*
Genes that were upregulated by MLC901				
ABHD12B	Abhydrolase domain containing 12B	2.42	ND1	24H
		2.15	ND2	12H
CCDC160	Coiled-coil domain containing 160	2.18	ND1	8H
		1.57	ND2	4H
CCL20	Chemokine (C-C motif) ligand 20	2.53	ND1	24H
		1.61	ND2	4H
CLEC18B	C-type lectin domain family 18, member B	1.68	ND1	4H
		2.39	ND2	12H
DOCK5	Dedicator of cytokinesis 5	2.58	ND1	4H
		1.56	ND2	4H
E2F8	E2F transcription factor 8	2.95	ND1	24H
		1.60	ND2	4H
FGF19	**Fibroblast growth factor 19**	4.06	ND1	24H
		3.45	ND2	24H
FOXQ1	Forkhead box Q1	3.29	ND1	12H
		1.73	ND2	12H
GALR2	**Galanin receptor 2**	2.10	ND1	8H
		1.66	ND1	12H
		2.29	ND2	24H
GPR64	**G protein-coupled receptor 64**	1.54	ND1	8H
		1.91	ND1	12H
		3.01	ND2	8H
ITGA1	**Integrin, alpha 1**	1.61	ND1	4H
		2.44	ND1	8H
		4.37	ND2	4H
LIMS2	LIM and senescent cell antigen-like domains 2	1.93	ND1	8H
		2.77	ND2	24H
LINC01330	Long intergenic non-protein coding RNA 1330	1.76	ND1	4H
		3.05	ND2	8H
		2.00	ND2	12H
MMP10	**Matrix metallopeptidase 10 (stromelysin 2)**	2.04	ND1	8H
		1.53	ND2	8H
NEURIM1	Neuronal integral membrane protein 1	1.95	ND1	24H
		2.59	ND2	24H
OCSTAMP	Osteoclast stimulatory transmembrane protein	1.66	ND1	4H
		1.80	ND1	8H
		3.97	ND2	12H
SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2	1.86	ND1	4H
		2.81	ND2	12H
SLCO2A1	Solute carrier organic anion transporter family, member 2A1	1.80	ND1	4H
		1.81	ND2	4H
SP5	Sp5 transcription factor	2.17	ND1	8H
		1.90	ND2	4H
		5.30	ND2	8H
SPANXA2	SPANX family, member A2	2.07	ND1	24H
		2.09	ND2	12H
TCEAL7	Transcription elongation factor A (SII)-like 7	1.51	ND1	24H
		1.52	ND2	8H
		2.09	ND2	12H
TDO2	**Tryptophan 2,3-dioxygenase**	1.62	ND1	4H
		2.38	ND1	24H
		4.51	ND2	8H
		1.55	ND2	24H
ZNF385C	Zinc-finger protein 385C	1.64	ND1	24H
		2.05	ND2	12H
Genes that were downregulated by MLC901				
ABCB4	ATP-binding cassette, sub-family B (MDR/TAP), member 4	−2.36	ND1	4H
		−1.58	ND1	12H
		−1.61	ND2	24H
ADAMTSL2	ADAMTS-like 2	−2.10	ND1	24H
		−1.94	ND2	24H
ANXA13	Annexin A13	−1.55	ND1	8H
		−2.06	ND2	12H
ELMOD1	ELMO/CED-12 domain containing 1	−5.93	ND1	8H
		−1.54	ND2	8H
FAM50B	Family with sequence similarity 50, member B	−1.56	ND1	12H
		−3.14	ND2	8H

Table 1. (Continued)

Gene symbol	*Description*	*Ave FC*	*Expt*	*Time point*
FGF3	**Fibroblast growth factor 3**	− 7.13	ND1	24H
		− 3.50	ND2	12H
LIMS2	LIM and senescent cell antigen-like domains 2	− 2.63	ND1	12H
		− 2.45	ND2	12H
LINC01330	Long intergenic non-protein coding RNA 1330	− 4.63	ND1	12H
		− 1.65	ND2	4H
		− 1.73	ND2	24H
LOC644612	Hypothetical protein LOC644612	− 2.25	ND1	8H
		− 3.08	ND2	8H
LOC645321	Hypothetical LOC645321	− 1.99	ND1	4H
		− 1.75	ND1	12H
		− 2.52	ND2	4H
LOC653524	Similar to tripartite motif-containing 16, transcript variant 1	− 1.64	ND1	8H
		− 4.02	ND1	24H
		− 1.59	ND2	4H
LOC100134006	Similar to ring finger protein 18, transcript variant 1	− 3.71	ND1	8H
		− 1.99	ND1	24H
		− 1.97	ND2	4H
SFMBT2	Scm-like with four mbt domains 2	− 2.96	ND1	12H
		− 1.54	ND2	12H
SYP	**Synaptophysin**	− 1.66	ND1	12H
		− 3.72	ND2	8H
		− 2.03	ND2	12H
ZAP70	Zeta-chain (TCR) associated protein kinase 70kDa	− 2.76	ND1	12H
		− 2.71	ND1	24H
		− 1.56	ND2	4H
ZCCHC12	Zinc finger, CCHC domain containing 12	− 4.13	ND1	12H
		− 1.51	ND2	4H
		− 1.56	ND2	12H

Genes in bold have associations to neural functions according to Gene Ontology and literature.

considered. There were 23 upregulated and 16 downregulated of such common genes upon NeuroAiD treatment during neural differentiation (Table 1), of which 10 upregulated genes (*SP5*, *TDO2*, *ITGA1*, *FGF19*, *FOXQ1*, *GPR64*, *NEURIM1*, *DOCK5*, *SLCO2A1* and *MMP10*) and eight downregulated transcripts (*ELMOD1*, *ZCCHC12*, *SYP*, *LOC644612, SFMBT2*, *LIMS2*, *LOC645321* and *ADAMTSL2*) were found changing within the same time point in both the experiments (Figures 3b and c and Table 1).

Fifty-five genes were found commonly regulated by MLC901 in all three independent experiments with the third performed in basal differentiation conditions

To ensure that the neural factors added in the NDM did not mask the effects of MLC901, we performed a third experiment whereby NDM free of BDNF, GDNF and antioxidants, referred to as basal NDM, was used for neural differentiation. There were 723 and 650 transcripts up- and downregulated, respectively, during neural differentiation by MLC901 in the absence of BDNF, GDNF and antioxidants (Figure 4a).

Of these differentially regulated genes, 29 upregulated and 26 downregulated genes were found common in all three independent experiments (Figure 4a). Among the common regulated genes, nine of them (*BIK*, *LINC01330*, *OCSTAMP*, *FGF19*, *GALR2*, *CCL20*, *MMP10*, *SPANXA2*, *ZNF385C*) were upregulated and eight (*FGF3*, *LOC100134006*, *ZAP70*, *TDO2*, *LIMS2*, *ADAMTSL2*, *FRAT1*, *ANXA13*) were downregulated with a fold change of more than 2 in at least one of the three experiments (Table 2). Of note, *FGF19* was found upregulated while *TDO2*, *LIMS2* and *ADAMTSL2* were downregulated at the same time points within the same gene in all three independent experiments (Figures 4b and c).

Genes having a role in neurogenesis and neural differentiation were found significantly regulated by MLC901 in all three independent experiments

To understand the biological significance of differentially expressed genes obtained from the expression profiling analysis, each list from the separate experiments were put through the web-based bioinformatics tool—The Database for Annotation, Visualization and Integrated Discovery (DAVID) v.6.7 to identify enriched gene ontologies associated with the genes. Functional annotations related to neurogenesis, brain development and transmission of nerve impulse were significantly enriched in all three independent experiments (Figure 5). Collectively these results suggest that MLC901 is modulating molecular pathways that are relevant to neurogenesis, which we will discuss in more detail below.

DISCUSSION

Our aim was to elucidate the effects of MLC901 on the neural induction of hESC to NPC and subsequent neural differentiation of the progenitors to neuronal and glial cells. These two stages of neural differentiation were investigated separately and cell states were assessed by immunofluorescence staining. Antibodies against stem cell marker, OCT4, and NPC markers, PAX6 and NESTIN, were used for the immunostainings to examine the rate as well as quantity of neural progenitors emerging upon neural induction in the absence and presence of 1 μg ml^{-1} MLC901 at days 1, 2, 3, 5 and 7. Similarly, immunostainings were performed at days 3, 7 and 14 on neural progenitors undergoing differentiation in the absence and presence of 1 μg ml^{-1} MLC901 using antibodies against neuronal markers—doublecortin, neuronal nuclei and microtubule-associated protein 2. There were no

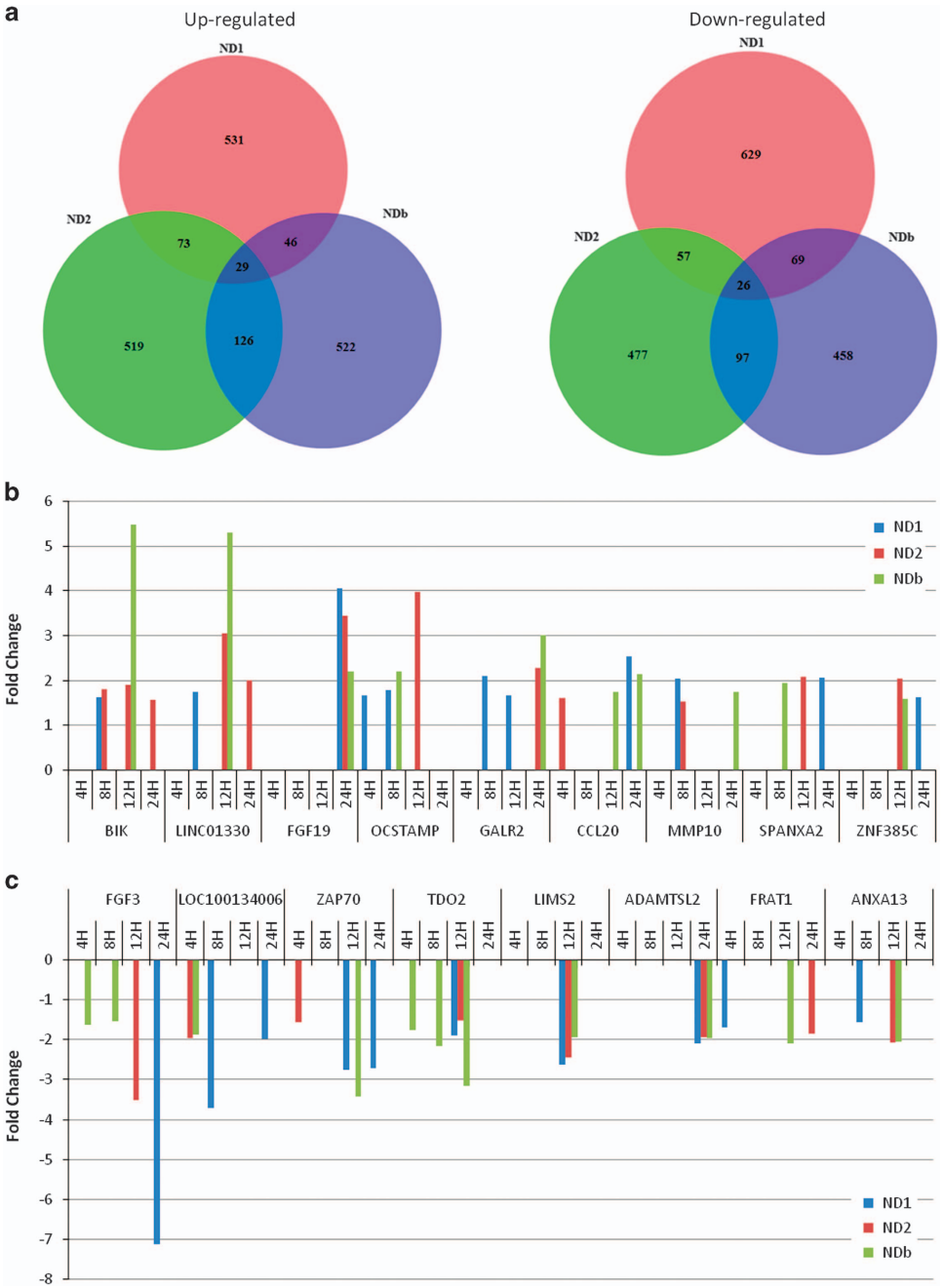

Figure 4. Genes differentially regulated by MLC901 in all three independent microarray experiments. (**a**) A Venn diagram of the number of genes differentially regulated (fold change >1.5 and P-value <0.05) by MLC901. There were 723 and 650 transcripts up- and downregulated, respectively, during neural differentiation by MLC901 in the absence of brain-derived neurotrophic factor, glial-derived neurotrophic factor and antioxidants. Detailed view of fold changes in gene expression: (**b**) 29 genes commonly upregulated and (**c**) 26 genes commonly downregulated (more than twofold in at least one of the experiments) by MLC901 in all three experiments. ND1, neural differentiation experiment 1; ND2, neural differentiation experiment 2; NDb, neural differentiation experiment 3 performed with basal differentiation medium.

obvious differences in the rate or level of expression of these progenitor or neuronal markers even at 10 μg ml^{-1} MLC901 (data not shown). For a more detailed and comprehensive view of molecular effects, we proceeded to look at differences in the gene expression levels influenced by MLC901 during the differentiation process using gene expression profiling. To this end, genome-wide microarray studies were performed on the human progenitors undergoing neural differentiation in the presence and absence of MLC901. This study was performed a second time with a differentiation media that was free of neural factors and antioxidants to eliminate any probability of these factors masking the true effects of MLC901.

In the first experiment, the neural progenitors were differentiated toward the neuronal lineage with specific neural factors such as BDNF and GDNF. Gene expression profiling showed that 23 genes were upregulated while 16 were downregulated more than twofold by MLC901. Of these, there were eight genes that have some neural associations—*FGF19*, *GALR2*, *GPR64*, *ITGA1*,

MMP10, *TDO2* (upregulated), and *FGF3* and *SYP* (downregulated) (Table 1). According to available information in the National Center for Biotechnology Information (NCBI) gene database, G protein-coupled receptor 64 (*GPR64*) is implicated in the neuropeptide signaling pathway while integrin, alpha 1 (*ITGA1*) is expressed in neuron projections and have axon guidance

Table 2. Genes found commonly influenced by MLC901 in all three independent experiments with fold change >2 in at least one experiment

Gene symbol	*Description*	*Ave FC*	*Expt*	*Time point*
Genes that were upregulated by MLC901				
BIK	BCL2-interacting killer (apoptosis-inducing)	1.64	ND1	8H
		1.82	ND2	8H
		1.90	ND2	12H
		1.58	ND2	24H
		5.48	NDb	12H
CCL20	Chemokine (C-C motif) ligand 20	2.53	ND1	24H
		1.61	ND2	4H
		1.76	NDb	12H
		2.14	NDb	24H
FGF19	**Fibroblast growth factor 19**	4.06	ND1	24H
		3.45	ND2	24H
		2.20	NDb	24H
GALR2	**Galanin receptor 2**	2.10	ND1	8H
		1.66	ND1	12H
		2.29	ND2	24H
		3.02	NDb	24H
LINC01330	Long intergenic non-protein coding RNA 1330	1.76	ND1	4H
		3.05	ND2	8H
		2.00	ND2	12H
		5.30	NDb	12H
MMP10	**Matrix metallopeptidase 10 (stromelysin 2)**	2.04	ND1	8H
		1.53	ND2	8H
		1.75	NDb	24H
OCSTAMP	Osteoclast stimulatory transmembrane protein	1.66	ND1	4H
		1.80	ND1	8H
		3.97	ND2	12H
		2.19	NDb	8H
SPANXA2	SPANX family, member A2	2.07	ND1	24H
		2.09	ND2	12H
		1.95	NDb	8H
ZNF385C	Zinc-finger protein 385C	1.64	ND1	24H
		2.05	ND2	12H
		1.59	NDb	12H
Genes that were downregulated by MLC901				
ADAMTSL2	ADAMTS-like 2	−2.10	ND1	24H
		−1.94	ND2	24H
		−1.95	NDb	24H
ANXA13	Annexin A13	−1.55	ND1	8H
		−2.06	ND2	12H
		−2.05	NDb	12H
FGF3	**Fibroblast growth factor 3**	−7.13	ND1	24H
		−3.50	ND2	12H
		−1.62	NDb	4H
		−1.53	NDb	8H
FRAT1	Frequently rearranged in advanced T-cell lymphomas	−1.69	ND1	4H
		−1.84	ND2	24H
		−2.08	NDb	12H
LIMS2	LIM and senescent cell antigen-like domains 2	−2.63	ND1	12H
		−2.45	ND2	12H
		−1.94	NDb	12H
LOC100134006	Similar to ring finger protein 18, transcript variant 1	−3.71	ND1	8H
		−1.99	ND1	24H
		−1.97	ND2	4H
		−1.88	NDb	4H
TDO2	**Tryptophan 2,3-dioxygenase**	−1.89	ND1	12H
		−1.52	ND2	12H
		−1.77	NDb	4H
		−2.17	NDb	8H
		−3.16	NDb	12H
ZAP70	Zeta-chain (TCR) associated protein kinase 70kDa	−2.76	ND1	12H
		−2.71	ND1	24H
		−1.56	ND2	4H
		−3.43	NDb	12H

Genes in bold have associations to neural functions according to Gene Ontology and literature.

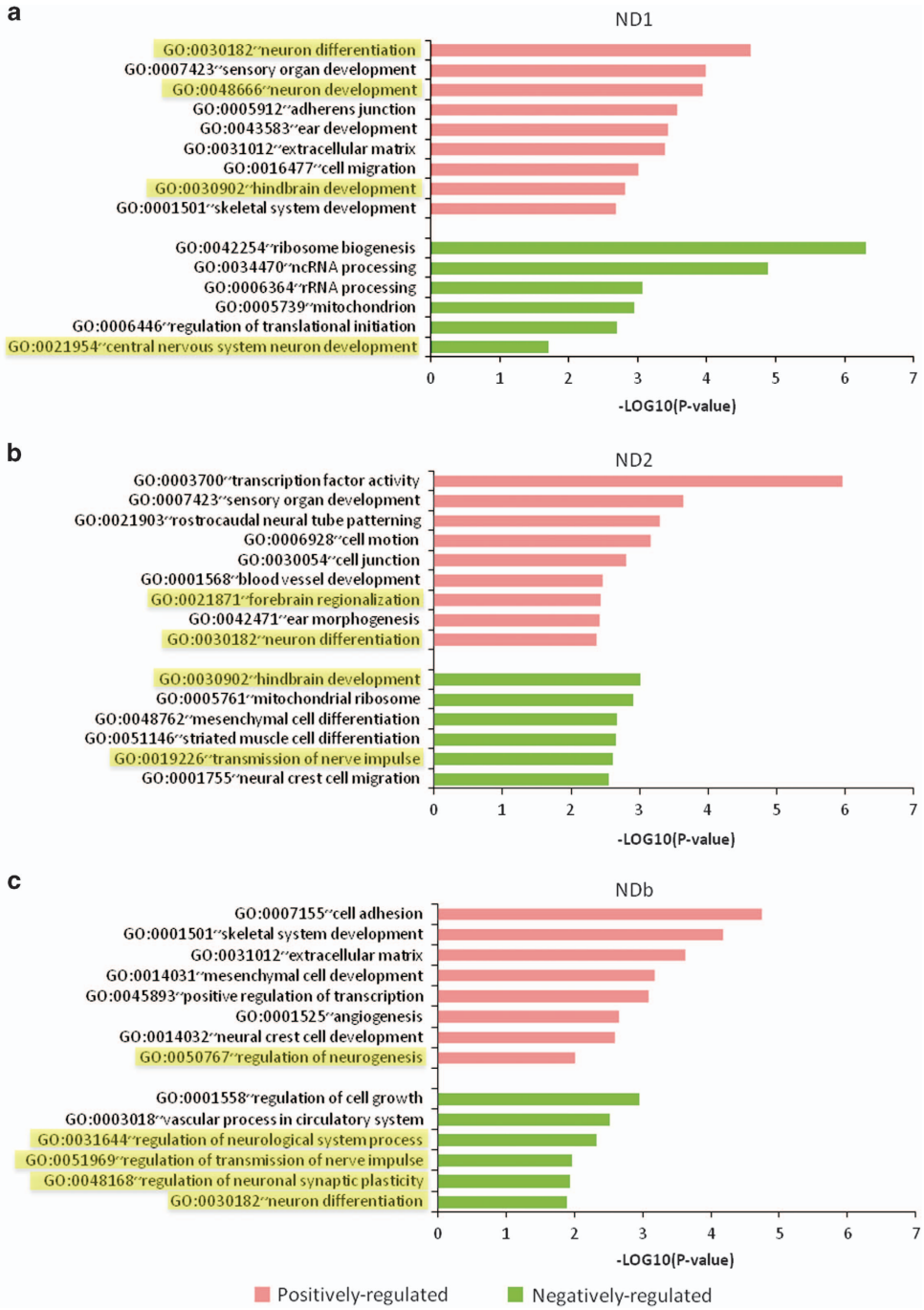

Figure 5. Top significantly enriched DAVID functional annotations associated with the genes differentially regulated by MLC901 in (**a**) neural differentiation experiment 1, (**b**) neural differentiation experiment 2 and (**c**) neural differentiation experiment 3 conducted with basal neural differentiation medium. Gene ontologies highlighted in yellow are related to neurogenesis.

functions. Synaptophysin (*SYP*) is expressed in neuron projections and excitatory synapse and is involved in the regulation of neuronal synaptic plasticity. As to why it was downregulated instead of being upregulated, it remains unclear. The remaining genes, *FGF19*, *GALR2*, *MMP10*, *TDO2* and *FGF3* will be discussed at length in the later part of the discussion as they were also found significantly regulated in the third experiment.

In the experiment performed with differentiation media devoid of any neural factors and antioxidants in the microarray analysis, we identified 55 common targets that were significantly (fold change >1.5; *P*-value <0.05) modulated in the same direction by MLC901 in both experiments despite a difference in the differentiation media conditions. Among the 55 common genes, 17 of them were differentially regulated more than 2-fold (Table 2). The consistent and robust regulation as well as the coherent functional annotations (Figure 5) of these differentially regulated genes by MLC901 in all three independent differential gene expression experiments gave us confidence in the authenticity of these 17 targets. Again, based on relevant findings in published literature and information in the NCBI gene database,

we found 5 targets, namely fibroblast growth factor 19 (FGF19), galanin receptor 2 (GALR2) and matrix metallopeptidase 10 (MMP-10), fibroblast growth factor 3 (FGF3) and tryptophan 2,3-dioxygenase (TDO2), with strong associations to functions related to neurogenesis and neuroprotection, which we would like to focus our discussions on.

Fibroblast growth factor (Fgf) signaling is highly implicated in the regulation of cell proliferation and differentiation in various parts of the brain during early mammalian development.[12] The relatively large Fgf family consists of 22 members[13] and FGF19 is a human orthologue of mouse Fgf15. Interestingly, it is expressed only in the human fetal brain and not in the adult brain.[14] Fgf19 was first discovered to be involved in cell proliferation and survival in the zebrafish forebrain, midbrain and cerebellum. In addition, it was found to have a role in the specification of gamma-aminobutyric acidergic interneurons and oligodendrocytes in the zebrafish forebrain.[15] Later studies revealed similar neural roles of Fgf19 in the avian and murine species. It was observed that avian FGF8, FGF10 and FGF19 promote statoacoustic ganglion neuron survival and neurite outgrowth[16] while murine Fgf15 promotes the neuronal differentiation of cortical progenitor cells and initiates the proper progression of dorsal midbrain neurogenesis in the mouse by controlling the expression of neurogenic and proneural transcription factors.[17,18] Furthermore, it was noted that human FGF19 promotes cell cycle exit of murine dorsal neural progenitors *in vitro*.[18] It could be postulated that MLC901 enhances the levels of FGF19 in the human neural progenitors to induce neural differentiation as well as neuronal survival.

The neuropeptide, galanin (Gal), plays a key role in neurite outgrowth and peripheral nerve regeneration as demonstrated by the decreased number of dorsal root ganglia neurons in Gal-knockout mice during development and the lack of peripheral nerve regeneration and neurite outgrowth in these mice upon induced nerve injury.[19] This effect of galanin was found to be mediated predominantly via one of the three G-coupled galanin receptors, GALR2. More recently, it has also been shown that galanin promotes neuronal differentiation, axonogenesis and dendritogenesis through GALR1 and GALR2 activation in neural stem cell cultures derived from the murine subventricular zone.[20] In addition to neurogenesis, galanin was also shown to have neuroprotective roles. The initial observation that galanin and its receptors were upregulated in brains associated with Alzheimer's disease and nerve injury led to subsequent studies in primary rat cortical neuronal cultures, which provided evidences that galanin exerts its neuroprotective function against amyloid-beta-induced neurotoxicity as well as nerve injury through the promotion of GALR2 expression.[21,22] Our data suggest that one of the possible neuroprotective mechanisms of MLC901 is to stimulate the expression of GALR2 in the human brain.

Notably, one of our targets, MMP-10, has been reported to be upregulated in human brains after stroke and its expression markedly reduced infarct size in a murine model of stroke, which makes it a promising profibrinolytic agent in the treatment of stroke.[23,24] Interestingly, nerve growth factor-induced upregulation of MMP-10 has also been implicated in improved dorsal root ganglia neurite outgrowth by increasing the proteolytic ability of the nerve cell to infiltrate into the extracellular matrix, thereby making way for the neurite.[25,26] Hence, MLC901 could enhance neurogenesis by boosting MMP-10 levels to aid neurite outgrowth during post-stroke recovery.

It was also observed that FGF3 was significantly downregulated by MLC901 during neural differentiation. Fgf signaling is critical in cell proliferation as well as differentiation in the brain.[12] The balance between the self-renewal of the neural stem cells and the transition to mature neurons is crucial in maintaining the right composition and size of the cortex and this requires the appropriate type and amounts of Fgfs and their receptors to interact at the precise developmental stages. It has been shown that depleting some of the Fgf receptors in the murine cortex reduces the number of neural precursors and shifts the uncommitted state to a more differentiated one, indicating that some Fgfs repress the maturing process of neural stem cells.[27] Although not stated explicitly in the study, one could postulate that FGF3 needs to be downregulated before the progenitor cells can progress towards corticogenesis and MLC901 could promote neural differentiation by downregulating FGF3 in the human NPCs.

Another noteworthy gene downregulated by MLC901 was *TDO2*. Elevated expression of TDO2, the first enzyme in the kynurenine pathway (KP) of tryptophan degradation, leads to the excessive formation of the KP metabolite, also known to be a neural excitotoxin, such as quinolinic acid, which is likely to be involved in the age-related neurodegenerative processes of Alzheimer's disease.[28] A reduction of the TDO2 enzyme, thought to be a metabolic regulator of α-synuclein, increased tryptophan levels and ameliorated the toxic effects of α-synuclein in the *Caenorhabditis elegans* model of Parkinson's disease.[29] Similarly, a genetic inhibition of the gene Tdo2 presented protection against neurodegeneration in a *Drosophila melanogaster* model of Huntington's disease.[30] It is still unclear how the reduction of TDO2 can help in stroke or traumatic brain injury but MLC901 could confer neuroprotective properties against age-related neurodegeneration by moderating the level of the TDO2 enzyme. However, what is confounding in the data is that in the first two experiments alone at time points 4, 8 and 24 h, TDO2 was upregulated by MLC901 (Figure 4b). It could be that in the first two experiments, in the context of a strong differentiation force, the TDO2 enzyme was required for other functions but in the case of the third experiment where the NPCs had to contend with the lack of antioxidants, MLC901 takes on a neuroprotective role by reducing the TDO2 enzyme to produce less neurotoxic by-products.

CONCLUSION

Our work is the first to employ a genome-wide platform to explore the effects of MLC901 on human neural progenitors during neurogenesis *in vitro*. From this work, we discovered some potential targets such as FGF19, FGF3, GALR2, MMP10 and TDO2 which MLC901 likely moderates in the human neural progenitors to bring about its neuroregenerative and neuroprotective properties. Armed with this knowledge, further work could be done to validate the targets by which MLC901 might act upon. These results were generated using *in vitro* derived neuronal cells. This approach provides a tractable experimental system to perform comprehensive gene expression analyses on human cells. However, it remains important to confirm these findings in *ex vivo* human tissues and animal models to confirm the pharmacogenomic responses to MLC901. Nonetheless, the insights gained here could help in the development of other therapeutic targets and applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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