MODULATING DNA METHYLATION FOR EFFECTIVE DIFFERENTIATION OF NEURAL STEM CELLS

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MODULATING DNA METHYLATION FOR EFFECTIVE DIFFERENTIATION OF NEURAL STEM CELLS

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LIST OF SYMBOLS AND ABBREVIATIONS

CNS	Central Nervous System
DNMT	DNA Methyltransferase
NSC	Neural Stem Cell
BDNF	Brain-derived growth factor
AD	Alzheimer's Disease
PCR	Polymerase Chain Reaction
APP	Amyloid Precursor Protein
5-azaC	5-azacytidine
SAM	S-Adenosylmethionine
FA	Folic Acid
NS	Neurosphere
DIV	"Days in Vitro"
DIFF	Differentiation Day
NSC-SR	Neural Stem Cell – Self-Renewal Culture Medium
NSC-DN	Neural Stem Cell – Differentiation Medium
PBS	Phosphate Buffered Saline
FBS	Fetal Bovine Serum

SUMMARY

Epigenetic mechanisms are pivotal for gene regulation and transcription, which are crucial to a proper functioning of the central nervous system (CNS). Numerous neurological and psychiatric diseases are caused by malfunctions in the CNS. Since epigenetics does not affect DNA sequencing and is reversible, it offers a very good method to treat different types of neurological diseases. DNA methylation is an essential process to the functioning of the CNS and is pivotal to gene expression. DNA methyltransferases (DNMTs) catalyze this process. Here, we analyze the impact of DNMT modulation on neural stem cell (NSC) differentiation into the different types of neural cells, neurons, astrocytes, and oligodendrocytes. Using DNA methylation inhibitors (5-azacytidine), the impact of regulation change on the differentiation of the NSCs is tested. We find that modulating DNA methylation effectively regulates NSC differentiation.

CHAPTER 1

INTRODUCTION

DNA Methylation

DNA methylation is a major mechanism for epigenetics and is crucial for regulating gene expression and development (Razin and Riggs, 1980). DNA methyltransferases (DNMTs) are responsible for DNA methylation, catalyzing the transfer of the methyl group from S-adenosylmethionine (SAM) to the position 5 of cytosine at CG sites in mammals. This process, when occurs at the gene promoter regions, causes the gene to be silenced (Biniszkiewicz et al., 2002; Feng et al., 2005; Liao and Karnik, 2015).

DNMT1, DNMT3a, and DNMT3b are the DNA methyltransferases present in mammals. DNMT1 is the major DNA methylation maintenance DNMT, while DNMT3a and DNMT3b are de novo DNMTs (Li et al., 1992) (Okano et al., 1999). DNMTs are dynamically regulated in the central nervous system (Feng et al., 2005). Previous studies have shown that overexpression or lack of expression of these DNA methyltransferases results in a variety of effects that range from cell death to overexpression of certain genes. For example, deletion of DNMT1 results in embryonic lethality and loss of DNA methylation whereas overexpression of DNMT1 causes hypermethylation and cell death (Biniszkiewicz et al., 2002; Li et al., 1992). Such studies suggest that different doses of DNMTs will have different effects in cells or organisms (Biniszkiewicz et al., 2002).

While the effects of DNMTs in many different settings have been analyzed, not much research has been focused on their role in differentiation of neural stem cells (NSCs) and in neuroengineering. Neural stem cells are lineage restricted stem cells that can be induced to differentiate into different types of neural cells: such as neurons, astrocytes, and oligodendrocytes. Neurons are responsible for the transmission of electrochemical signals whereas astrocytes are glial cells with multiple functions, such as the support of endothelial cells outlining the blood-brain barrier, providing nutrients to the nervous tissue and repairing damaged tissue in the brain. Lastly, oligodendrocytes are responsible for outlining the axon of a neuron with myelin, which causes the action potentials to increase in speed. This project will focus on evaluating the impact of modulating DNMT activity in NSC differentiation on neuronal and glial cell differentiation.

In this study, we use the compound 5-azacytidine (5-azaC), a DNMT small molecule inhibitor, to modulate the activity of DNMTs. 5-azaC was first synthesized by Piskala and Sorm (1964), originally developed and tested as a nucleoside antimetabolite with clinical specificity for acute myelogenous leukemia (Čihák, 1974; Šorm et al., 1964). It is a pyrimidine nucleoside analogue of cytidine with antitumor activity. Azacytidine is incorporated into DNA, where it binds to DNA methyltransferases and inhibits DNMTs from catalyzing the transfer of the methyl group from S-adenosylmethionine (SAM) to the position 5 of cytosine at CG sites in mammals, thereby blocking DNA methylation (Biniszkiewicz et al., 2002; Feng et al., 2005; Liao and Karnik, 2015).

While DNMT1 is required for neural stem cell differentiation in adult mice (Noguchi 2015), 5AzaC has been shown to facilitate neuronal and astrocytic differentiation from neural stem cells in cell culture (Majumder et al., 2013; Schinstine and Iacovitti, 1997). 5-azaC in combination with brain-derived growth factor (BDNF) cooperate to product more mature neurons from neural stem cells, and 5-azaC in combination with HDAC inhibitor induces astrocytic differentiation from human neural stem cells (Majumder et al., 2013; Schinstine and Iacovitti, 1997). In this study, using mouse neural stem cells, I analyze the effects of 5-azaC treatment on neural network formation.

Neural Stem Cells Growth and Differentiation

Neural stem cells (NSCs) are stem cells that are located in brain and give rise to specialized cell types in the central nervous system. They play a pivotal role in the growth of the brain during development. In an adult brain, NSCs reside in the subventricular zone (SVZ) of the brain. Stem cells have the ability to both self-renew and differentiate into several more specified cell types. Unlike embryonic stem (ES) cells that can undergo differentiation in numerous types of cells, NSCs can only differentiate into three different types: neurons, astrocytes, and oligodendrocytes. During development of the nervous system, NSCs differentiate into neurons first prior to differentiating into glial cells such as astrocytes and oligodendrocytes (Bull and Bartlett, 2005; Clarke, 2003; Zhang et al., 2012).

Neural stem cells cultured with EGF (Epidermal Growth Factor) and FGF (Fibroblast Growth Factor) aggregate to form large spherical neurospheres. These heterogeneous groups of

cells can be mechanically dissociated and propagated. Only a small percentage of the NSCs inside the neurospheres remain as NSCs capable of forming neurospheres. These neurospheres can propagate and be induced to differentiate into the different types of neural cells present in the brain.

The effects of altering DNMT activities on the culture of neurospheres and the differentiation of these neural stem cells are investigated in this study. First, a neural differentiation protocol was developed and optimized. Second, the NSCs were treated with small molecules, 5-azaC, that inhibit DNA methylation, and their neural network formation upon differentiation was analyzed.

MATERIALS AND METHODS

NSC Culture and Differentiation

NSCs were isolated from E12.5 mouse embryo brains and cultured in Neural Stem Cell Self Renewal culture medium (StemCell Technologies, CA) to form neurospheres in 6-well UltraLow attachment plates (Corning Cat#3471). 500K cells were seeded per well. This amount was counted by adding Trypan blue. Every 2-3 days, ½ media was changed and the neurospheres were dissociated through mechanical pipetting.

These neurospheres were cultured until there were enough cells for differentiation assays (50K cells per well of 24-well plate (Corning)). They were subsequently induced to form neural networks by culturing in Neural Stem Cell Differentiation culture medium (StemCell Technologies, CA) following protocol as shown in Figure 1 on plates pre-treated with 200mg/ml Laminin (Thermo Fischer Scientific) for 3-4 hours. The media were changed every 3-4 days. The formation and progression of neural networks were observed and imaged every two days starting on DIFF2 by phase-contrast microscopy.

5-azaC treatments

Treatment of the NSCs with 5-azacytidine (Sigma-Aldrich) was performed following established procedure. NSCs are cultured in NSC-SR medium using above-mentioned procedure until there are 2.5x10(6) cells per well. Four treatments using the following concentrations and durations: T1 (0.5 uM 5-azaC + 2 days), T2 (2 uM 5-azaC + 2 days), T3 (0.5 uM 5-azaC + 4 days), and T4 (2 uM 5-azaC + 4 days), were compared.

Immunofluorescence

Immunostaining was performed following previously established procedure (Zhang et al., 2012). Briefly, the cells were cultured and induced to differentiate on glass cover slips. On each chosen day (DIFF2, DIFF4, DIFF8, and DIFF12), the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 min, followed by permeabilization with 0.2% Triton-X-100 (Sigma-Aldrich) for 20 min. Cells were subsequently washed with PBS 2x5min and incubated overnight at 4°C in 10% FBS/PBS solution. The following primary antibodies were used: anti-GFAP (1:300) (Abcam); anti-Nestin (1:30) (Millipore), and anti-β-Tubulin-III (1:100) (Sigma). The following secondary antibodies were used: Cy3-couple donkey anti-rabbit antibody (1:200) (Jackson) and Alexa Fluor 488-couple donkey anti-mouse antibody (1:200) (Jackson). Nuclei were counter stained with Hoescht. During the process of immunostaining, the antibodies were diluted in 10% FBS/PBS in which the fixed and permeabilized cells incubated for 1 hour both times. During both staining processes, the cells were washed 3x5min with PBS. Images were collected on an Olympus Fluorescence Microscope using 100X magnification.

.

RESULTS

NSCs were isolated from E12.5 mouse embryo brains and cultured in Neural Stem Cell Self Renewal culture medium (StemCell Technologies, CA) to form neurospheres in 6-well UltraLow attachment plates (Corning Cat#3471). To develop an effective neural network formation assay, several culture conditions were compared. An optimized protocol for the growth and differentiation of NSCs was established as shown in Figure 1. In addition, I tested different seeding densities of NSCs and identified optimal conditions for NSC and NS culture for formation of robust neural networks as shown in Figure 2.

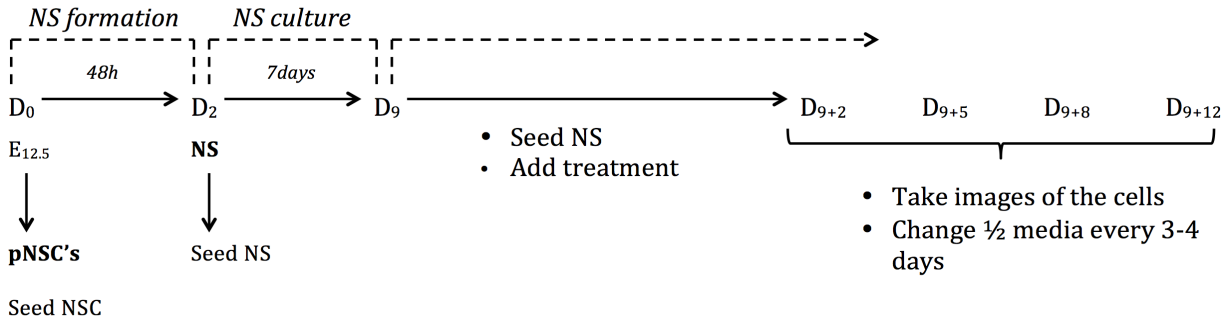

Figure 1. Culture and differentiation scheme of neural stem cells. NSCs isolated from mouse fetal brain were cultured to form neurospheres (NS) and differentiated into neural networks.

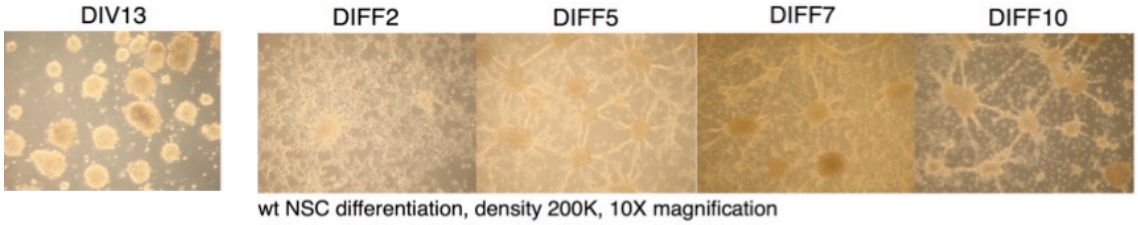

Figure 2 Differentiation timeline starting from DIV 13.

Cells were treated with 5-azacytidine to determine how inhibition of DNA methylation would affect differentiation and formation of neural networks. Four different treatments were applied on the undifferentiated cells prior to differentiation. The four treatments used were: 0.5 ug/ml for 2 days (T1), 2 ug/ml for 2 days (T2), 0.5 ug/ml for 4 days (T3), and 2 ug/ml for 4 days (T4). Results from cell counting after 4 days of culture/treatment showed that higher dosage and prolonged treatments (four days) led to significant cell death in the culture (Figure 3).

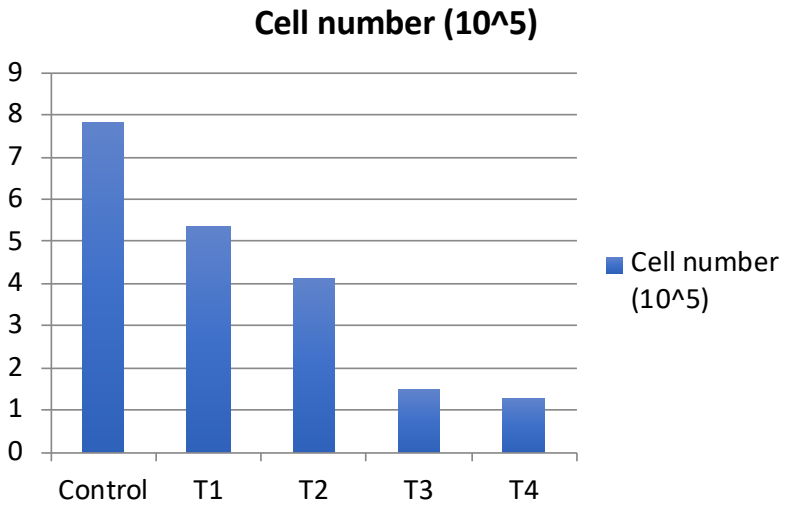

Figure 3 Graph showing decrease in cell viability following treatment with 5-azaC.

Dramatic differences in NS morphologies with different 5-azacytidine treatments on NSC culture were observed. 5-AzaC treatment inhibited NS growth as shown by smaller neurospheres in treated NSC culture compared to the untreated cells (Figure 4). After AzaC treatment, cells were counted and 50k cells per well were seed on 24-well plate for NSC differentiation assay. Cell count and pictures showed that a higher concentration of 5-azacytidine in prior treatment continued to cause cell death in differentiating culture. Treatments with longer periods of time (T3 days (T1 and T2) levered long lasting and strong neural networks whereas the treatments

lasted 4 days (T3 and T4) resulted in large amounts of cell death and poor differentiation (Figure 5). Upon differentiation, cells pre-treated with T1 and T2 conditions showed even more robust networks than that of the untreated cells. At day 10 post differentiation (DIFF10), the neural networks formed in untreated cells started to degenerate whereas the networks in cell culture pre-treated with 5'-AzaC for 2 days were sustained. In contrast, cells pre-treated for 4 days (T3 and T4) never formed networks and died very quickly. The poor differentiation in T3 and T4 treated cultures may be partly due to the toxic effects from prolonged treatment with 5-azaC. These results indicate dynamic effects of inhibition of DNA methylation on NSC differentiation and neural network formation.

Immunostaining was performed on the differentiated cells. Images were taken on DIFF2, DIFF4, DIFF8, and DIFF12. Three different antibodies used to immnuostain the cells were: anti-Nestin, anti-β-tubulin-III and anti-GFAP. Nestin is a neural stem cell marker, β-tubulin-III is a neuronal cell marker and GFAP is a marker for astrocytes. Preliminary results show that the expression of Nestin is decreased accompanied by the increase of β-tubulin-III and GFAP expression during NSC differentiation (data not shown).

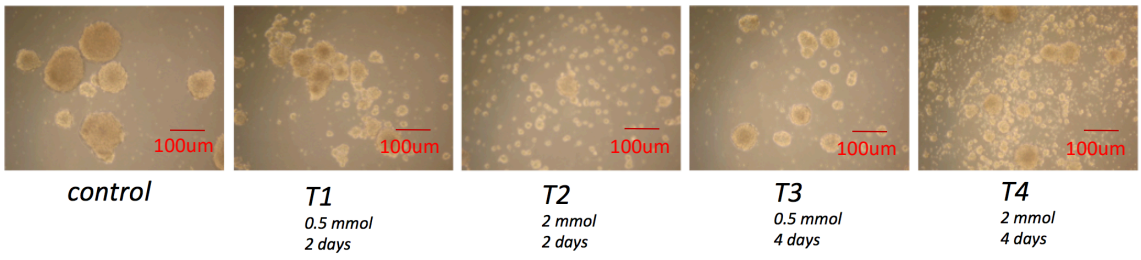

Figure 4 Effects of 5-azacytidine treatments on neural stem cell culture

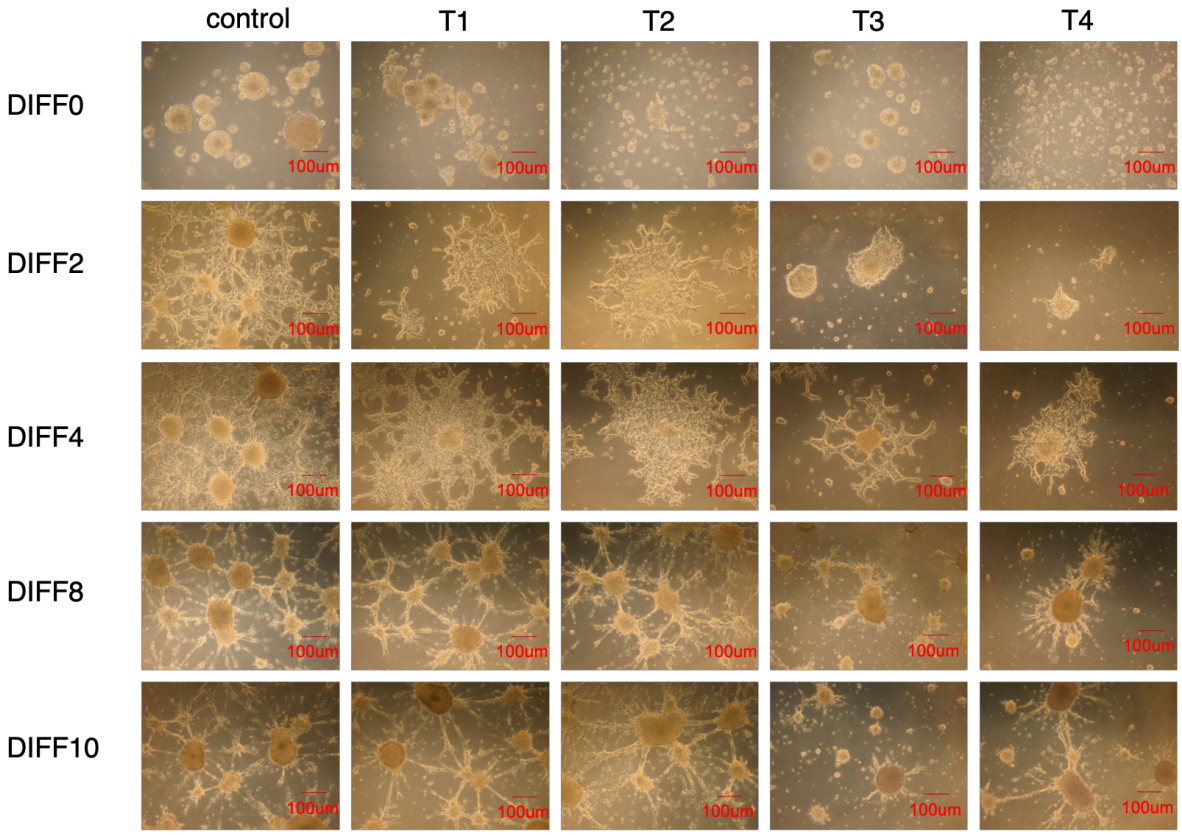

Figure 5 Differentiation of 5-azacytidine treated neural stem cells

DISCUSSION

This study shows that treatment of neural stem cells with DNA methylation inhibitor, 5-AzaC, alter the differentiation process, suggesting that modulation of DNA methylation in NSCs dramatically affect NSC differentiation. An increase in neural network strength was observed after neural stem cells treated with 0.5 μg/ml and 2 μg/ml for 2 days were differentiated. Not only was the network visibly stronger, but the differentiated cells also sustained longer than the untreated control cells. In contrast, when the cells were treated for 4 days, there was a significant increase in cell death and the network would dissolve soon after differentiation was induced. Preliminary studies suggest that the expression of GFAP was severely increased in cells treated with 5-azacytidine for 2 days (data not shown). This suggests that the percentage of astrocytes present in these neural networks is increased with 2-day treatment of 5-azacytidine. This may partially contribute to a better neural network observed in these cultures.

FUTURE WORK

Immunostaining of the differentiating culture with neuronal marker and glial markers will provide further evidence and quantitation of neurons and glial cells produced from the differentiation culture. In addition, gene expression profiling by RNA sequencing of the differentiating cells at different treatments and time points (DIFF2, 4, 8, and 12) will help assess how the gene expression program is affected by 5-AzaC treatment during differentiation.

CONCLUSION

The results from this study indicate dynamic effects of inhibition of DNA methylation on NSC differentiation as well as neural network formation. While low dose, short treatment with 5-AzaC improves neural differentiation, high dosage treatment appears to undermine differentiation, probably due to toxic effects of 5-AzaC.

REFERENCES

Biniszkiewicz, D., Gribnau, J., Ramsahoye, B., Gaudet, F., Eggan, K., Humpherys, D., Mastrangelo, M.-A., Jun, Z., Walter, J., and Jaenisch, R. (2002). Dnmt1 Overexpression Causes Genomic Hypermethylation, Loss of Imprinting, and Embryonic Lethality. Molecular and Cellular Biology *22*, 2124-2135.

Bull, N.D., and Bartlett, P.F. (2005). The adult mouse hippocampal progenitor is neurogenic but not a stem cell. The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience *25*, 10815-10821.

Čihák, A. (1974). Biological effects of 5-azacytidine in eukaryotes. Oncology *30*, 405-422.

Clarke, D.L. (2003). Neural stem cells. Bone Marrow Transplant *32*, S13-S17.

Feng, J., Chang, H., Li, E., and Fan, G. (2005). Dynamic expression of de novo DNA methyltransferases Dnmt3a and Dnmt3b in the central nervous system. Journal of Neuroscience Research *79*, 734-746.

Li, E., Bestor, T.H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell *69*, 915-926.

Liao, J., and Karnik, R. (2015). Targeted disruption of DNMT1, DNMT3A and DNMT3B in human embryonic stem cells. *47*, 469-478.

Majumder, A., Dhara, S.K., Swetenburg, R., Mithani, M., Cao, K., Medrzycki, M., Fan, Y., and Stice, S.L. (2013). Inhibition of DNA methyltransferases and histone deacetylases induces astrocytic differentiation of neural progenitors. Stem Cell Res *11*, 574-586.

Okano, M., Bell, D.W., Haber, D.A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell *99*, 247-257.

Razin, A., and Riggs, A.D. (1980). DNA methylation and gene function. Science *210*, 604.

Schinstine, M., and Iacovitti, L. (1997). 5-Azacytidine and BDNF enhance the maturation of neurons derived from EGF-generated neural stem cells. Exp Neurol *144*, 315-325.

Šorm, F., Piskala, A., Čihák, A., and Veselý, J. (1964). 5-Azacytidine, a new, highly effective cancerostatic. Cellular and Molecular Life Sciences *20*, 202-203.

Zhang, Y., Cooke, M., Panjwani, S., Cao, K., Krauth, B., Ho, P.-Y., Medrzycki, M., Berhe, D.T., Pan, C., McDevitt, T.C.*, et al.* (2012). Histone H1 Depletion Impairs Embryonic Stem Cell Differentiation. PLoS Genetics *8*, e1002691.