

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

**Transcriptional and epigenetic program of embryonic stem cell  
differentiation into neurons by retinoic acid**

by Zoltán Simándi

Supervisor: Prof. Dr. László Nagy



UNIVERSITY OF DEBRECEN  
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

DEBRECEN, 2014

# **Transcriptional and epigenetic program of embryonic stem cell differentiation into neurons by retinoic acid**

By Zoltán Simándi, MSc.

Supervisor: Prof. Dr. László Nagy, MD, PhD, DSc, MHAS

Doctoral School of Molecular Cell and Immune Biology, University of Debrecen

Head of the **Examination Committee:** Prof. Dr. János Szöllősi, PhD, DSc  
Members of the Examination Committee: Dr. Péter Nagy, MD, PhD, DSc  
Dr. Ágota Apáti, PhD

The Examination takes place at Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen at 13:00 p.m., 10th November, 2014

Head of the **Defense Committee:** Prof. Dr. János Szöllősi, PhD, DSc  
Reviewers: Dr. Ágota Apáti, PhD  
Dr. Shaun M. Cowley, PhD  
Members of the Defense Committee: Prof. Dr. Imre Boros, PhD, DSc  
Dr. Péter Nagy, MD, PhD, DSc

The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 13:00 p.m., 12th of January, 2015.

## **INTRODUCTION**

### **Levels of gene expression regulation**

The human genome encodes approximately 25,000 genes. Many genes, known as housekeeping genes, are needed by almost every type of cells in the body. However, most of the genes are expressed in a tissue-specific manner. How these genes are switched on and off at the correct time and in the correct place is a crucial and enduring question for researchers. Tissue specific expression of the genes is controlled at multiple levels. Regulation of transcription in eukaryotes is mainly a result of the combined effects of the (1) chromatin structure and (2) the interactions of transcription factors and co-regulators.

The fundamental structural unit of chromatin is the nucleosome which consists of DNA wrapped around an octamer of histones. Posttranslational modifications of histones, such as acetylation, phosphorylation, methylation and ubiquitination, constitute an important mechanism of gene regulation. Histones can be modified at many sites in the same time. Enzymes have been identified for the vast majority of histone modifications. Histone acetyltransferases (HATs), such as CBP/P300, are enzymes that acetylate conserved lysine amino acids on histone proteins and results in increased gene expression. The reversal of acetylation is catalyzed by histone deacetylases (HDACs) and believed to correlate with transcriptional repression. Histones can be also methylated by the transfer of methyl group from the methyl donor S-adenosyl methionin (SAM) to lysine or arginine residues by methyltransferases.

Function of non-histone proteins is also regulated by the aforementioned posttranslational modifications. Beside the well established role of acetylation and phosphorylation, recent research has implicated arginine methylation as a major regulator of cellular processes, including transcription, translation, nucleocytoplasmic transport, signalling, DNA repair, RNA processing and splicing.

## **Arginine methylation and chromatin remodeling**

Arginine methylation is a prevalent posttranslational modification found on both cytoplasmic and nuclear proteins, including the histones. Arginine methylation is catalyzed by a family of Protein aRginine MethylTransferases (PRMTs). Members of the PRMT family can be grouped based on their enzyme activity into three groups. Type I enzymes (PRMT1, 2, 3, 4, 6 and 8) catalyze the formation of asymmetric dimethylarginine (aDMA) residues while the Type II (PRMT5) enzyme catalyze the formation of symmetric dimethylarginine (sDMA) residues. Both Type I and II enzymes generate monomethylarginine (MMA) intermediates. The Type III enzyme (PRMT7) only generates a MMA mark.

Arginine methylation has been shown to regulate nuclear export and import, protein-protein interactions, ribosome biogenesis, pre-mRNA splicing, transcriptional elongation and transcription. Moreover, arginine methylation also regulates cell cycle checkpoints and the DNA damage response. PRMT1 is broadly expressed and it is believed to be the dominant mammalian arginine methyltransferase. Several substrates of PRMT1 has been identified, including hnRNP molecules, Sam68 and Mre11. Disruption of PRMT1 gene in mice result in embryos die shortly after implantation, failing to develop beyond E6.5.

It has been well established that PRMT1 is an H4-specific arginine methyltransferase and that methylation of histone H4 Arg 3 could result in transcriptional activation. Studies with isolated histones have shown that pre-methylation of H4R3 by PRMT1 stimulates acetylation by P300 and thus enhance transcriptional activation. However, it is unclear how PRMT1 is targeted to specific promoters or transcriptional regulatory regions.

Less is known about the biological function of PRMT8, a vertebrate-restricted paralogue of PRMT1. PRMT8 shares more than 80% sequence identity with PRMT1 but in contrast to the wide distribution of PRMT1, PRMT8 expression is restricted to the central nervous system. Recent data suggest that PRMT8 may play important roles non-overlapping with PRMT1 in embryonic and neural development depending on its specific N-terminus.

## **Retinoic acid signaling and the retinoic acid receptors**

Transcription factors are one of the most common tools that our cells use to control gene expression. The nuclear receptor superfamily are ligand-activated transcription factors that play diverse roles in cell differentiation, development, proliferation, and metabolism and are associated with numerous pathologies such as cancer, cardiovascular disease, inflammation, and reproductive abnormalities. One of the best understood example for nuclear receptor-mediated transcriptional regulation is the retinoic acid signaling pathway, where the ligand for the receptors is the vitamin A derivative retinoic acid (RA). Retinoic acid is not produced by all the cells of the body, however cells without active RA synthesis still may have access to it from the environment. The intracellular level of RA is controlled through its metabolism. In the cytoplasm RA binds to intracellular retinoic acid binding protein 2 (Crabp2) and delivered into the nucleus. In the nucleus, RA binds to and activate the Retinoic Acid Receptor : Retinoid X Receptor (RAR:RXR) heterodimer. RARs and RXRs exhibit the conserved structure of nuclear receptors, including DNA-binding domain (DBD) and ligand binding domain (LBD). Liganded RAR:RXR heterodimers bind to specific regulatory DNA element, called RA response element (RARE). *In vitro* binding and transactivation studies demonstrated that RAR:RXR preferentially bind to direct repeat (DR) sequences of the hexameric motif (A/G)G(G/T)TCA, often spaced by 5 or 2 nucleotides (DR5 and DR2, respectively).

## **Epigenetic regulation of the retinoic acid receptors**

As stated above, in the absence of ligands RAR:RXR heterodimer is located in the nucleus and bind to DNA response elements of target genes. According to a recent model, the unliganded receptors recruit co-repressor complexes that contain histone deacetylases (HDACs), which in turn induce a repressive chromatin state at the target locus. Ligand

binding triggers an allosteric change that lead to the exposure of RAR:RXR surface for interaction with co-activators (CBP/P300, NCoA, etc.).

### **Retinoic acid in cellular differentiation and neurogenesis**

Retinoic acid is one of several molecules that pattern vertebrate embryos by forming an anterior to posterior (A–P) concentration gradient. Possible functions of RA in embryogenesis were first inferred by studying its excess and absence during embryonic development. These studies also indicated the essential role of RA in neurogenesis. The wide distribution of different RA receptors throughout the central nervous system strongly suggest that retinoid signaling is necessary for neural development and proper brain function. In the developing neural tube, retinoid signaling initiates neural differentiation, controls patterning and differentiation of spinal motor neurons, and specifies caudal hindbrain and rostral cervical spinal identity. Retinoid signaling also plays an important role in the developmental patterning of the hindbrain through the regulation of the Homeobox (Hox) genes.

Despite the valuable information obtained in these *in vivo* models, our understanding are still limited in terms of the mechanistic details and molecular events of retinoid signaling pathway. Cellular model systems, such as embryonic stem cells can help us to better understand the mechanistic details how RA coordinate the cell fate decisions.

### **Embryonic stem cells as a model system of cellular differentiation**

Embryonic stem cells (ESCs) are pluripotent cells isolated from the inner cell mass of blastocysts at day 3.5. ESCs differentiate *in vitro* into almost all kind of cells and the development of neuronal cells from mouse ESCs has been especially established. Studies using ESCs have begun to unravel the network of cytokines and transcription factors responsible for their maintenance of pluripotency and proper cell differentiation. Aggregates of ES cells resemble normal embryonic development much closer than previously thought,

and provide a valuable *in vitro* model for studying the effects of various factors on cell lineage decisions in early stages of embryonic development.

The area of understanding neurogenesis by modeling it with the differentiation of ESCs has been particularly active, due to the great promise of treating variety of neurological diseases and central nervous system injuries. Several examples confirmed that reproducing *in vivo* inductive and patterning events *in vitro* efficiently directs the differentiation of ESCs into selected neural derivatives. Signals, such as retinoic acid, FGFs, Wnt and Dkk are known to be expressed in a concentration gradient from the posterior of the embryo to the anterior and play indispensable role in the patterning of vertebrate embryonic nervous system. Application of these inductors in a temporal manner resulted in various neural cell type specification from ESCs.

## AIMS

Our study focused on the transcriptomic and epigenetic events of retinoic acid induced differentiation of mouse embryonic stem cells.

The general aim was to understand the following concepts in transcriptional regulation of embryonic development and cellular differentiation:

1. gene selective transcriptional regulation
2. fine-tuning of signal responsiveness
3. cell-type specific interpretation of the same signal
4. functional consequences of signal misinterpretation

As specific goals, we aimed to:

1. Determine components of retinoic acid signaling that are expressed in undifferentiated embryonic stem cells
2. Identify retinoic acid induced genes and describe their epigenetic regulation
3. Identify putative cross-talk between the two co-regulator, P300 and PRMT1
4. Characterize PRMT1-dependent transcriptional events in cellular differentiation
5. Establish a multistage differentiation model of mouse embryonic stem cells to neurons to explore the mechanistic and functional role of novel co-regulator PRMT1 and PRMT8 in neurogenesis.

## **MATERIALS AND METHODS**

### **Embryonic stem cell culture**

Embryonic stem cells (kind gift of Tomo Saric and Istvan Szatmari) were grown on a layer of mitotically inactivated mouse primary embryonic fibroblasts (PMEF) or SNL feeder cells to promote growth and prevent differentiation. Cell cultures were incubated in 5% CO<sub>2</sub> at 37°C. The ESC medium was prepared by supplementing DMEM Glutamax media with 15% FBS (Hyclone), 1000U of leukemia inhibitory factor (LIF), penicillin/streptomycin, non-essential amino acids and 2-mercaptoethanol.

### **Neural differentiation of stem cell cultures**

ESCs were cultured on 0.1% gelatin coated plates in feeder-free condition for at least three passages prior to differentiation. Embryoid body formation was induced by plating  $4 \times 10^6$  cells onto bacteriological Greiner Petri dishes in 15 ml ES differentiation media (10% FBS). Medium was complemented with retinoic acid in final concentration 5  $\mu$ M from day 5 to day 8. EBs were disaggregated by 0.05% trypsin at day 8 and cells were plated onto Poly-L-ornithin (PORN)-laminin pre-coated culture dishes in N2 medium (DMEM, F-12, L-glutamine, insulin, transferrin, selenite, putrescine, progesterone, BSA) at about  $2 \times 10^5$  cells per cm<sup>2</sup> density.

### **shRNA-based stable gene silencing**

shRNA lentiviral plasmids (MISSION shRNA, TRCN0000018490-493 and TRCN0000097479-483) were purchased from Sigma for targeting the mouse PRMT1 and PRMT8, respectively. Lentiviruses were produced by transient co-transfection of shRNA lentiviral plasmids and helper plasmids (pMD2.G, pRSV-Rev, pMDLg/pRRE) into 293T. Transduced cells were then maintained under puromycin selection. Non-target shRNA was used as a control.

### **Plasmid and transient transfection**

Full-length wild-type human PRMT1 was kind gift of Henry MF. NHf-290-Hoxb1-luciferase and 2xDR2-luciferase plasmids were kind gift of T Ogura and RM Evans. Luciferase reporters were generated by insertion of PCR amplified enhancer region of Hoxb1, Cyp26a1, Pmp22, Spsb1 or PRMT8. Full length human PRMT8 was PCR amplified from GFP-PRMT8 (kind gift of M. Bedford) and inserted into EcoRI/SalI site of pCMV-Tag2 (Stratagene). All constructs were verified by DNA sequencing. Transfection was carried out using FuGENE transfection reagent (Promega), according to the manufacturer's instructions. Luciferase activity was determined by Luciferase Assay System (Promega) and normalized to  $\beta$ -galactosidase activity.

### **Real-time quantitative PCR (RT-qPCR)**

Total RNA was isolated with TRIZOL reagent (Invitrogen). cDNA synthesis was performed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's recommendation. Quantitative PCR was performed using real-time PCR (ABI PRISM 7900, Applied Biosystems). Gene expression was quantified by the comparative  $C_T$  method and normalized to Gapdh expression.

### **Microarray analysis**

Microarray experiments were performed by the UD-GENOMED Medical Genomic Technologies Ltd. GeneChip Mouse Gene 1.0 ST Arrays were used and data analysis was performed using GeneSpring GX 12.6 software (Agilent, Santa Clara, CA). Affymetrix data files were imported to the GeneSpring by RMA16 algorithm. Normalization to the median of control samples was performed. Differential expression analysis was carried out to determine the number of changing entities by using unpaired T-test, Asymptotic p-value computation and Benjamini-Hochberg multiple testing correction. Hierarchical cluster analysis was

performed using Euclidean distance metric. Data were analyzed through the use of IPA (Ingenuity® Systems, [www.ingenuity.com](http://www.ingenuity.com)).

### **RNA-seq**

Illumina RNA-sequencing was performed using standard procedures at the Centre National de Genotypage (CNG) Paris, France. RNA-Seq library was prepared from two biological replicates by using TruSeq RNA Sample Preparation Kit (Illumina) according to manufacturer protocol. Analysis of RNA-seq data has been carried out at the Center for Clinical Genomics and Personalized Medicine, University of Debrecen by Attila Horvath. The TopHat-Cufflinks-CummeRbund toolkit trio were used for mapping spliced reads, making transcript assemblies, and getting, sorting and visualizing gene expression data. Downstream data analysis were performed in R. Expression Heatmap was created using R package pheatmap.

### **Western blot analysis**

20 µg protein whole cell extract was separated by SDS-gelelectrophoresis in 10 or 12.5% polyacrylamide gel and then transferred to Immobilon-P Transfer Membrane (Millipore Crp., Billerica, Massachusetts). Membranes were probed with anti-PRMT1 (07-404; Millipore), anti-PRMT8 (ab73686; Abcam), anti-ASYM24 (07-414; Millipore), anti-SYM11 (07-413; Millipore), anti-OCT3/4 (sc5279; Santa Cruz), anti-ACTIN (A2066, Sigma), anti-GAPDH (sc32233; Santa Cruz) antibodies, according to the manufacturer's recommendations.

### **Immunofluorescence detection of stem cell and differentiation markers**

Cells were fixed in 4% paraformaldehyde and incubated for 1h in blocking buffer (PBS, 10% FBS, and 0.1% TritonX-100). Primary antibodies were applied overnight at 4°C. After three washes in PBS, FITC conjugated secondary antibodies were diluted at 1: 200 in blocking

buffer and applied for 1h at room temperature. Cells were washed three times in PBS and visualized on an AxioVision inverted fluorescence microscope. Primary antibodies were obtained from the following sources: anti-OCT3/4 (sc-5279; Santa Cruz), anti-SSEA (MC-480; DSHB), anti-TUJ1 (MMS-435P; Covance), anti-PRMT1 (07-404; Millipore), anti-PRMT8 (HPA039747; Sigma), anti-H4R3me2a (Active Motif, Cat.no 39705), anti-SYN1 (Millipore, AB1543P and BD Biosciences Cat.no 611393).

### **Chromatin immunoprecipitation**

Embryonic stem cells were cultured in the presence or absence of RA for various time points and then cross-linked in two steps with disuccinimidyl glutarate (DSG) (ProteoChem) and 1% methanol-free ultrapure formaldehyde (Thermo Scientific, #28908). Chromatin was sonicated with Diagenode Bioraptor and immunoprecipitated with antibodies against pre-immune IgG (Millipore, 12-370), RXR (Santa Cruz, sc-774), RAR (Santa Cruz, sc-773), P300 (Santa Cruz, sc-585), HDAC3 (Santa Cruz, sc-11417), Oct3/4 (Santa Cruz, sc-8628), H3K27ac (Abcam, ab4729) and H4R3me2a (Active Motif, Cat.no 39705). Library preparations for sequencing has been carried out at the Center for Clinical Genomics and Personalized Medicine, University of Debrecen. ChIP-seq libraries were prepared by Ovation Ultralow Library Systems (Nugen) or TruSeq ChIP sample preparation kit according to manufacturer's instructions. Primary analysis of ChIP-seq data has been carried out at the Center for Clinical Genomics and Personalized Medicine, University of Debrecen by Erik Czipa, Gergely Nagy and Endre Barta using the ChIP-seq analyze command line pipeline. Genome coverage files for visualization were made by makeUCSCfile.pl. Integrative Genomics Viewer (IGV2.3, Broad Institute) was used for data browsing. Numbers of overlapping peaks were visualized by VennMaster. HOMER was used for making meta-histograms, and finding motif enrichments.

### **Calcium imaging, loose-patch and whole-cell patch-clamp recording**

ESCs derived neurons were loaded with the calcium indicator dye. Calcium imaging measurements were carried out at the Department of Physiology, University of Debrecen using a Zeiss Axioskop microscope. For extracellular loose-patch recording of the neuronal action potential firing, micropipettes filled with artificial cerebrospinal fluid were used. Electrical activity of the cells was recorded in voltage-clamp mode using an Axopatch 200A amplifier. Data acquisition was performed at 10-kHz sampling rate. For whole cell patch clamp recordings patch pipettes with 5 M $\Omega$  pipette resistance were pulled with a Narishige vertical puller, and filled with a solution containing (in mM): K-gluconate, 120; NaCl, 5; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10; EGTA, 2; CaCl<sub>2</sub>, 0.1; Mg-ATP, 5; Na<sub>3</sub>-GTP, 0.3; Na<sub>2</sub>-phosphocreatinine, 10; biocytin, 8; pH 7.3. Whole-cell patch-clamp recordings were performed using an Axopatch 200A amplifier, with 10 KHz sampling rate.

### **Tissue samples**

Glioblastoma and normal brain tissue were collected during neurosurgical operations in the Department of Neurosurgery, University of Debrecen. Each sample was collected from a different patient. Normal samples were collected either during functional neurosurgery for epilepsy or non-tumor herniated brain tissue during tumor surgery. All procedures were approved by the National Ethical Committee, and every patient signed an informed consent form.

### **Statistical analysis**

RT-qPCR and ChIP-qPCR data were conducted as biological triplicates. Values are expressed as mean  $\pm$  SD of the mean. GraphPad Prism version 5.02 was used for data interpretation. We performed unpaired t-tests and results were considered to be significant with  $p < 0.05$ .

## **RESULTS**

### **Expression pattern of elements of retinoic acid signaling in undifferentiated ESCs**

As a first step of our work we identified which elements of the retinoid signaling pathway are present in undifferentiated ESCs. Our global gene expression analyses showed that only cellular retinoic acid binding protein 1 (Crabp1), retinoic acid receptor gamma (Rarg) and retinoid X receptor alpha and beta (Rxr $\alpha$ , Rxr $\beta$ ) were expressed at higher levels in ESCs. Retinol binding proteins (RBPs), alcohol dehydrogenases (ALDHs) and Cyp26s were present at very low mRNA levels. These results suggest that undifferentiated ESCs are unable for endogenous production of RA.

### **Identification of retinoic acid induced genes**

We carried out RNA-seq experiments in order to identify target genes regulated by retinoic acid in embryonic stem cells. Only 35 genes, including Cyp26a1, Dhars3, Dleu7, Hoxa1, Hoxb1, Rar $\beta$ , Stra6, Stra8 were detected as statistically significantly regulated genes following short-term retinoic acid treatment.

### **Retinoic acid dependent epigenetic changes**

We used the previously described retinoic acid response elements of Cyp26a1 and Hoxa1 as representative examples for further epigenetic characterization. Using chromatin immunoprecipitation coupled with qPCR we determined the ligand-induced dynamics of the RAR and RXR receptors, and also the co-repressor HDAC3 and co-activator P300 bindings at the enhancer regions of these genes. However RAR and RXR were enriched in both two cases upon retinoic acid treatment, we could not detect the binding of P300 or HDAC3 at any time point on the enhancer of Cyp26a1, suggesting the existence of an enhancer-specific epigenetic regulation in the retinoic acid induced transcriptional signaling.

### **Characterization of retinoic acid-dependent P300 cistrome in mouse ESCs**

Genome-wide analysis of P300 binding allowed us to identify 3420 consensus P300 binding sites present in untreated ESCs. Motif analysis revealed that in undifferentiated ESCs P300 is mainly recruited to genomic regions enriched for stem cell specific transcription factor Oct3/4, Sox2, Klf4, Esrrb and Nanog motifs. Upon RA treatment there was a ~3 fold reduction in the total number of P300 binding sites. Combination of P300 ChIP-seq with the retinoic acid regulated gene list obtained in the RNA-seq analysis confirmed the gene selective recruitment of P300 to retinoic acid response elements. Based on this observation we classified the genes as P300-independent (eg. Cyp26a1, Dleu7, Stra8) or P300-dependent (eg. Hoxa1, Hoxb1, Pmp22). Interestingly, analysis of published Oct3/4, Nanog and Sox2 ChIP-seq data obtained in undifferentiated ESCs revealed that P300-occupied enhancers also were enriched for at least one of the stem cell-specific transcription factors, showing the characteristics of the so called regulatory "hot spots".

### **Identification of PRMT1 as a selective co-repressor of "regulatory hotspots"**

Crosstalk between protein arginine methyltransferases (PRMTs) and P300 was demonstrated previously. Gene expression data obtained from RNA-seq in undifferentiated ESCs revealed the high level expression of PRMT1, suggesting its dominant role in ESC's asymmetric arginine methylation. To understand the contribution of PRMT1-mediated arginine methylation to P300-mediated transcriptional and epigenetic program of ESCs, we established stable PRMT1 knockdown cells using lentiviral-based approach.

Genome-wide comparison of gene expression in RA-treated cells revealed an altered retinoic acid responsiveness in PRMT1-depleted cells. While most of the established retinoid targets, such as Cyp26a1, Dhrr3, Dleu7, Rbp1, Stra8 were induced to the same degree in PRMT1-depleted and control cells, Hoxa1, Hoxb1, Stra6, Pmp22 and Spsb1 showed higher induction in hypomethylated cells. The selective effect of PRMT1 on retinoic acid induced gene

expression and the results obtained in previous analysis of P300 binding showed remarkable overlap, indicating a putative crosstalk between PRMT1 and P300 in the regulation of many retinoic acid induced genes.

### **Differentiation potential of PRMT1-depleted embryonic stem cells**

Differentiation of ESCs is often induced by cell aggregation resulting in the formation of embryoid bodies (EBs). This spontaneous differentiation of wild-type ESCs induced significant changes in the expression level of approximately 1000 genes. Genome-wide comparison and RT-qPCR validation of spontaneously differentiated control and PRMT1-depleted cells showed that classical lineage markers of endoderm, mesoderm and ectoderm were similarly induced upon spontaneous differentiation. Injection of PRMT1 *knockout* ESCs into immunodeficient mice resulted in teratoma formation with obvious differentiated structures from all three germinal layers, excluding the possibility that residual PRMT1, present in knockdown cells, is sufficient for differentiation.

### **Asymmetric arginine methylation is present at distinct stages of retinoic acid induced neural differentiation**

Having established that PRMT1 affected retinoic acid responsiveness of Hox genes that are essential for the proper neural differentiation, we next specifically focused on the role of PRMT1 in neural differentiation. We set up an embryonic stem cell-based model system of neural differentiation that resulted in terminally differentiated neurons with electrophysiological properties similar to those brain-derived neurons.

Proteins with asymmetric dimethylated arginine residues were present in ESCs. Asymmetric arginine methylation level of these proteins was changing dynamically during retinoic acid induced neural differentiation, suggesting either an increased level of target proteins or overall Type I enzyme-activity.

PRMT1 was highly expressed in ESCs and remained constant during neural differentiation showing a slight increase upon RA-treatment. Other Type I PRMTs were less abundant in ESCs, but PRMT2, PRMT6 and PRMT8 showed significantly increased gene expression in differentiated neurons.

### **PRMT8 is retinoic acid induced target gene in differentiating embryoid bodies**

Comparison of the expressional profile of PRMTs upon short-term RA-induction of day 4 EBs revealed the early upregulation of PRMT8. PRMT8 expression could be induced by retinoic acid and the RAR specific ligand AM580, but RXR specific LG268 had no effect. Bioinformatic analysis revealed that promoter region of PRMT8 contains a direct repeat with no spacer (AGGTCAAGGTCA, DR0), that can bind RAR:RXR. Transfecting an enhancer trap vector that contained this ~300bp genomic region of the PRMT8 promoter, we could validate functionally the element in response to RA treatment in ESCs. These results confirm PRMT8 as a direct RA-regulated gene.

### **PRMT8 act as a co-activator of retinoic acid signaling**

Based on the sequence similarity of PRMT1 and PRMT8, PRMT8 was also thought to act as a co-regulator of retinoic acid signaling. Enhancer trap vector of Hoxb1 RARE transfected with the reporter alone showed RA-dependent induction, which was further stimulated by co-transfection of PRMT8 expression plasmid. Depletion of PRMT8 in ESCs resulted in a decreased signal intensity, which could be restored by the overexpression of PRMT8.

### **PRMT1 and PRMT8 regulates large set of genes in neural cells**

To study if loss of PRMT1 and/or PRMT8 has consequences on retinoic acid induced neuronal differentiation or gene expression, stable knockdown cells were differentiated. At day 12 of differentiation the knockdown-derived neural cells showed similar morphology and

high expression of typical neural markers, such as Lhx1, Pax6 or Tuj1 to wild-type cells. Genome-wide comparison of cells at day 16 revealed dysregulation of several genes (947) in PRMT1 or PRMT8-depleted neurons.

### **PRMT1 and PRMT8 are present in one complex and regulate genes relevant in neurological diseases**

Grouping of the differentially expressed genes identified that a large fraction of the genes (473 out of 947) showed similar dysregulation in both knockdown cell types, suggesting that they possess similar biological functions. Co-immunoprecipitation studies confirmed that the two proteins are likely present in one complex. Ingenuity pathway analysis of the 473 genes implicated PRMT1 and PRMT8 in neuronal synaptic formation, glutamate receptor signaling and axonal guidance. Detailed analysis of the dysregulated genes also suggested that loss of PRMT1 and PRMT8 might be involved in the development of various neurological disorders.

### **Loss of PRMT8 results in PRMT1-independent transcriptional changes**

Further analysis of the gene expression data revealed that many genes, such as Cxcr4, Dhfr or Efemp1, previously linked to glial differentiation and gliomagenesis, were expressed differentially only in PRMT8 knockdown cells. Using PRMT1-PRMT8 double knockdown cells we found that these genes are indeed PRMT8-dependent, but not affected significantly by PRMT1.

### **Loss of PRMT8 is a marker of glioblastoma multiforme**

Prompted by the finding that PRMT8 might regulate genes associated with astrocytoma and glioblastoma, we used a large number of human primary glioma samples to further investigate the role of PRMT8 in the development of glioma. Our measurements confirmed that PRMT8-dependent Cxcr4 and Efemp1 are indeed dysregulated in glioblastoma multiforme (GBM).

PRMT8 itself also showed a substantially lower expression level in GBM samples, while PRMT1 did not show a difference between the disease and control groups. These data indicate that loss of PRMT8 and genes regulated by it are putative markers in GBM and might participate in its development.

## **DISCUSSION**

### **Retinoic acid signaling in embryonic stem cells**

It has become well established that retinoids and retinoid signaling has an essential role and impact on ESCs differentiation and lineage commitment. Retinoids are now widely used as inducers of certain differentiation pathways, however early transcriptomic and epigenetic events of this signaling pathway remained largely uncharacterized. Our global gene expression and epigenetic analyses provide a more detailed map of this pathway and the contribution of retinoid signaling to pluripotency and lineage-specific differentiation. It appears that retinoic acid receptors are present in the ESCs while the enzymes involved in processing of retinoic acid are largely absent. Genome-wide transcriptomic analysis helped us to characterize the early target genes that are induced upon short-term retinoic acid treatment. The low number of genes that is regulated early upon induction is somewhat surprising. However, several important transcription factor are included in the list. A likely scenario, that retinoic acid turns on master regulators (such as members of the Hox-cluster) as the first wave of ligand-response and these early induced factors will be later on responsible for the downstream effect and dramatic changes observed phenotypically in the culture.

### **Epigenetic events of retinoic acid induced gene expression**

Characterization of receptor and co-regulator binding upon retinoic acid treatment led us to question the validity of the recent accepted model of retinoic acid induced transcription. Unexpectedly, we found that in ESCs RAR:RXR binding is rather inducible than static. We also observed that P300, which was so far accepted as a general co-activator of RAR:RXR signaling pathway, show enhancer and gene selectivity. Selectivity is likely provided by the cell-type specific transcription factors bound to the close proximity of these regions in ESCs. This example of cell-type specific transcription factor-driven enhancer selective co-regulator

binding suggest a novel mechanism how a cell-type specific transcriptional response might be achieved.

### **P300 and PRMT1 are selective regulators of retinoic acid mediated signaling**

We also identified PRMT1 as a novel regulator of P300-dependent retinoic acid responsiveness. PRMT1 has been previously identified as a ubiquitously expressed secondary co-activator for nuclear receptors. It has been also shown to bind the activation domain (AD2) of primary co-activators and enhance transcription. In contrast to this, our results now show a repressive function of PRMT1, confirming in principle the findings of previous studies providing evidence for co-repressor roles for PRMT1 in different cellular context. Important to note that similar phenomenon has been reported in case of other co-activators, thus this opposing roles of PRMT1 in gene expression regulation are not unique.

Gene and enhancer selectivity is also a novel and striking feature of PRMT1, however the exact mechanism remained unclear. Strikingly, PRMT1-sensitive sites show characteristics of cell-type specific regulatory “hot spots”: key transcription factors, such as Oct3/4, Sox2, Klf4 and Nanog are enriched and mark these genomic regions. Importantly, the co-activator P300 is also selectively recruited to these sites upon RA-treatment, providing further evidence to the existence of distinct epigenetic states between PRMT1-sensitive and insensitive sites. Since we found no indication that loss of PRMT1 would affect P300 recruitment to these “hot spots”, a potential mechanism is that P300 or any regulatory member of the protein complex is arginine methylated by PRMT1 and this modification in turn affects the co-activator function of the P300 complex. This possibility would also explain why only P300-recruiting genomic regions are PRMT1-sensitive. Indeed, we found that PRMT1-dependent arginine methylation of certain proteins of the P300 complex are detectable, suggesting an indirect crosstalk between P300 and PRMT1.

## **Functions of PRMT1 and PRMT8 in neurogenesis**

A previous comparison of mammalian PRMTs revealed that PRMT1 and PRMT8 share the highest degree of identity within this enzyme family. Our results identified PRMT8 as a direct RA-regulated arginine methyltransferase that also contribute to retinoic acid responsiveness by acting as a co-activator of the retinoid signaling. The role of PRMT1 and PRMT8 in the regulation of retinoic acid responsiveness raised the question, whether loss of PRMT1 and/or PRMT8 has any consequences on retinoic acid induced neuronal differentiation or gene expression. Genome-wide analysis of differentiated neurons revealed dysregulation of several genes in PRMT1 or PRMT8-depleted cells and these genes could be linked to various functional defects. Interestingly, dysregulation of similar genes could be detected in both knockdown cell types.

It is an intriguing question whether PRMT1 and PRMT8 are involved in pathological conditions of the brain as well. Our results showed that loss of PRMT8 resulted in a decrease in the level of *Gfra2* and increase in the level of *Cxcr4*, *Dhfr* and *Efemp1*. These are established markers of astrocyte-derived glioma. We used a large patient cohort and found that the RNA levels of PRMT8 were almost completely down-regulated in glioma tissues. A trivial explanation may be that PRMT8, a highly specific neural marker, is not expressed in astrocyte-derived tumor. Alternatively, loss of PRMT8 positively affects astrocyte differentiation, resulting in a shift in cell fate commitment. Although further work is required, these results already provide strong support to the notion that PRMT8 might be a genetic risk factor in gliomagenesis and also a putative therapeutic target. Regardless of the mechanism, our results implicate loss of PRMT8 as a biomarker of glioma tissues.

## SUMMARY

We combined genetic approaches with genome-wide gene expression technologies to unravel the retinoic acid signaling in mouse embryonic stem cells and the contribution of P300, PRMT1 and PRMT8 to *in vitro* retinoic acid induced neuronal differentiation. We propose that there are two distinct phases during the course of neural differentiation: in early stages PRMT1 acts as a selective repressor of a large set of retinoic acid induced genes (Hoxa1, Hoxb1, Pmp22 and Spsb1). The promoter regions of these PRMT1-sensitive genes are regulatory “hot spots” as these sites are also occupied by Oct3/4, Sox2, Klf4 or Nanog and show P300 recruitment upon RA-treatment. This way PRMT1 arms the cells with a negative-feedback mechanism to limit RA’s effect on a subset of target genes.

Subsequently, the RA signal directly induces the expression of PRMT8. In this late stage, PRMT8 collaborate with PRMT1, but PRMT8 might also exist in complexes which do not contain PRMT1. PRMT8 in such complex acts as a co-activator that potentiates retinoid response. This novel mechanism suggest that PRMT1 and its closest paralogue PRMT8 integrate the morphogenic RA signal in a temporal manner. Loss of PRMT1 or PRMT8 results in mostly similar changes in neural specification, but PRMT1 and PRMT8 have independent regulatory potential as well.



Register number: DEENKÉTK/381/2014.  
Item number:  
Subject: Ph.D. List of Publications

Candidate: Zoltán Simándi  
Neptun ID: E0Z9HS  
Doctoral School: Doctoral School of Molecular Cell and Immune Biology  
MTMT ID: 10040517

### List of publications related to the dissertation

1. **Simándi, Z.**, Czipa, E., Horváth, A., Kőszeghy, Á., Bordás, C., Póliska, S., Juhász, I., Imre, L., Szabó, G., Dezső, B., Barta, E., Sauer, S., Károlyi, K., Kovács, I., Hutóczky, G., Bognár, L., Klekner, Á., Szűcs, P., Bálint, B.L., Nagy, L.: PRMT1 and PRMT8 regulate retinoic acid dependent neuronal differentiation with implication to neuropathology.  
*Stem Cells*. "accepted by publisher" (2014)  
IF:7.133 (2013)
2. **Simándi, Z.**, Cuaranta-Monroy, I., Nagy, L.: Nuclear receptors as regulators of stem cell and cancer stem cell metabolism.  
*Semin. Cell Dev. Biol.* 24 (10-12), 716-723, 2013.  
DOI: <http://dx.doi.org/10.1016/j.semcdb.2013.10.002>  
IF:5.971
3. **Simándi, Z.**, Bálint, B.L., Póliska, S., Rühl, R., Nagy, L.: Activation of retinoic acid receptor signaling coordinates lineage commitment of spontaneously differentiating mouse embryonic stem cells in embryoid bodies.  
*FEBS Lett.* 584 (14), 3123-3130, 2010.  
DOI: <http://dx.doi.org/10.1016/j.febslet.2010.05.052>  
IF:3.601





### List of other publications

4. Cuaranta-Monroy, I., **Simándi, Z.**, Kolostyák, Z., Doan-Xuan, Q., Póliska, S., Horváth, A., Nagy, G., Bacsó, Z., Nagy, L.: Highly efficient differentiation of embryonic stem cells into adipocytes by ascorbic acid.  
*Stem Cell Res.* 13 (1), 88-97, 2014.  
DOI: <http://dx.doi.org/10.1016/j.scr.2014.04.015>  
IF:3.912 (2013)
5. Czimmerer, Z., Hulvely, J., **Simándi, Z.**, Varallyay, É., Havelda, Z., Szabó, E., Varga, A., Dezső, B., Balogh, M., Horváth, A., Domokos, B., Török, Z., Nagy, L., Bálint, B.L.: A versatile method to design stem-loop primer-based quantitative PCR assays for detecting small regulatory RNA molecules.  
*PLoS One.* 8 (1), 10 p., 2013.  
IF:3.534
6. **Simándi, Z.**, Nagy, L.: Retinoid signaling is a context-dependent regulator of embryonic stem cells.  
In: Embryonic Stem Cells - Differentiation and Pluripotent Alternatives. Ed.: Michael S. Kallos, InTech - Open Access Publisher, Rijeka, 55-78, 2011.

Total IF of journals (all publications): 24,151

Total IF of journals (publications related to the dissertation): 16,705

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

17 November, 2014



## LIST OF PRESENTATIONS

1. Annual meeting of the Biochemical Society (Szeged, aug. 30-sept.3, 2008)  
Title: A PRMT1 szerepének tanulmányozása egér embrionális összejték neurális differenciációjában (in hungarian)
2. 2nd Molecular Cell and Immune Biology Winter School (Krompachy, Slovakia, Jan. 6-9, 2009)  
Title: New research objects and tools: ES and iPS
3. 3rd Molecular Cell and Immune Biology Winter School (Mariazell, Austria, Jan. 7-10, 2010)  
Title: Role of PRMT1 in retinoid signaling during stem cell differentiation
4. 4th Molecular Cell and Immune Biology Winter School (Galyatető, Jan. 11-14, 2011)  
Title: Retinoid signaling in embryonic stem cell differentiation
5. In focus the stem cell research (Debrecen, March 10-11, 2011)  
Title: Arginine methylation in stem cell derived neurons
6. 5th Molecular Cell and Immune Biology Winter School (Galyatető 2012. jan. 4-7)  
Title: The nuclear receptor coactivator PRMT1 is a context-dependent repressor
7. 75th anniversary of Albert Szent-Györgyi's Nobel prize award (Szeged, March 22-25, 2012)  
Title: PRMT1 and 8 control cell fate specification of differentiating embryonic stem cells via selectively tuning retinoid-induced gene expression
8. 6th Molecular Cell and Immune Biology Winter School (Galyatető, Jan. 8-11, 2013)  
Title: Genes and development
9. 23rd Wilhelm Bernhard Workshop (Debrecen, aug 19-23, 2013)  
Title: PRMT1 and PRMT8 coordinately regulates retinoic acid-driven differentiation of mouse embryonic stem cells
10. Annual meeting of the Biochemical Society (Debrecen, aug 24-27, 2014)  
Title: PRMT1 and PRMT8 regulate retinoic acid dependent neuronal differentiation with implications to neuropathology
11. MTA - Bioinformatics in brain research (Budapest, November 11, 2014)  
Title: *In vitro* neuron, *in silico* PRMT8, *in vivo* glioma (in hungarian)

## ACKNOWLEDGEMENT

I would like to extend thanks to the many people, who helped and supported me throughout my studies.

This thesis would not have been possible without my supervisor Dr. Laszlo Nagy.

I am grateful to Prof. Laszlo Fesus and Prof. Jozsef Tozser, the former and recent heads of the Department of Biochemistry and Molecular Biology for the opportunity to work in a well-equipped environment with intelligent, dedicated colleagues.

I owe my deepest gratitude to Balint L. Balint who was my first tutor and introduced me to stem cells and epigenetics.

I am also thankful to the many collaborators who not only just provided additional data but I could really learn from them: Gabor Szabo, Laszlo Imre, Almos Klekner, Tibor Hortobagyi, Miklos Antal, Peter Szucs, Aron Koszeghy, Endre Barta, Erik Czipa, Istvan Szatmari, Mate Demeny.

I am thankful to all the past and present members of the Nuclear Receptor Research Group for their help, scientific discussions. Special thanks to Ixchelt Cuaranta-Monroy, Szilard Poliska, Bence Daniel, Gergely Nagy, Attila Pap, Zsolt Zimmerman, Attila Horvath, Peter Brazda.

I should acknowledge the endless help I received from technicians: Marta Beladi, Beata Szalka, Ibolya Furtos and Tímea Cseh.

I should acknowledge the help I received from my talented undergrad students, Zsuzsanna Kolostyak and Zsanett Sari.

I also wish to express my deepest gratitude to my beloved family for their help and support. And to the dearest person in my life, Kata, for your understanding, generosity. And still I cannot promise you that there will be no more weekends sacrificed for the sake of science :)