Rybp is required for neural differentiation of mouse embryonic stem cells

Ph.D. thesis

Gergő Kovács

Supervisor:

Dr. Melinda K. Pirity

Institute of Genetics,

Biological Research Center of the Hungarian Academy of Sciences

Szeged

2016.

INTRODUCTION

Neural differentiation of embryonic stem cells

Every specialized cell type of an adult body develops from pluripotent embryonic stem cells. During central nervous sytem development, in the early phase, pluripotent stem cells differentiate into multipotent neural stem cells, then into neural progenitor cells. In the late phase of neural differentiation, these progenitors develop terminally differentiated neurons, astrocytes and oligodendrocytes. This complex process has several steps and their disturbance may cause neurological developmental disorders. The processes of neural induction are controlled by transcription regulators. Among these, polycomb repressive complexes are responsible of maintaining pluripotent stem cell state and consequently initiating differentiation by de-repressing the key developmental regulators. My thesis focused on one of these polycomb repressive complex members, namely the Ring1 and Yy1 Binding protein (Rybp) and its role in neural lineage commitment of mouse embryonic stem cells.

Rybp is essential for embryonic development and central nervous system development *in vivo*

Our group previously showed that *rybp null* mutant mice died at implantation and in *rybp* heterozygous mice there were severe neurodevelopmental disorders, including neural tube defects and exencephaly. Since the early embryonic lethality of the *rybp null* mutant mice obscures the genetic analysis of neural lineage commitment *in vivo*, we utilized an *in vitro* stem cell cell based system to investigate the role of Rybp during neural differentiation. My work was based on a remarkable feature of stem cells that under proper *in vitro* conditions can retain their pluripotency and differentiation capacity as well. By using *in vitro* neural differentiation model system, we could investigate the spatiotemporal differentiation events at various neural developmental stages in the absence of Rybp.

AIMS

The main goal of this thesis was to characterize the role of Rybp in neural lineage commitment *in vitro*.

The detailed aims of this study were:

- to examine whether the *rybp null* mutant mouse embryonic stem cells are capable to differentiate into all major neural lineages (neuron, oligodendrocyte, astrocyte).
- to characterize the properties of the progenitor cells derived from *rybp null* mutant mouse ES cells, especially with regard to their ability to balance self-renewal and differentiation during neural development.
- to determine which cell types have impaired differentiation capability as a result of Rybp absence.
- to find candidate downstream target genes of Rybp that have changed their expression depending on the presence or absence of Rybp during neural differentiation.

METHODS

In vitro neural differentiation

Mouse wild type (*rybp*$^{+/+}$) and *rybp null* mutant (*rybp*$^{-/-}$) embryonic stem cells were cultured on gelatin coated dishes. Neural differentiation of embryonic stem cells was induced as *Bibel et al* described, with some modifications. The cells were seeded at identical initial density into surfaces where cell attachment was prevented, so they were allowed to aggregate in suspension and formed embryoid bodies for 4 days. In order to facilitate induction of neuroectoderm, retinoic acid was added to the medium and the embryoid bodies were cultured for 4 days further, then they were dissociated and plated onto surface-treated dishes at identical density. The plated cells form neural culture for a further 6 days. The cells were harvested for further analyses at different time points of neural differentiation: days 0, 3, 7, 10 and 14.

Morphological analysis

We performed morphological analysis of the differentiating cell cultures at different timepoints (see above). The stem cells (day 0) and the embryoid bodies (day 3, day 7) were fixed with 4% paraformaldehyde, the neural cultures (day 10, day 14) were stained with cresyl violet. Images were obtained using Olympus cellR microscope. The fixed embryoid bodies were sectioned then stained with hematoxylin-eosin to visualize the histological structures.

Molecular analysis

We performed relative gene expression analysis and protein expression level analysis of the differentiating cells in the marked timepoints. For relative gene expression analysis quantitative real-time PCR assays were performed. Relative gene expression changes of pluripotency factors, polycomb repressive complex members, germline markers and neural marker genes were quantified during *in vitro* neural differentiation in both cell lines. For protein expression analysis immunocytochemistry was performed, where we got information of the spatiotemporal distribution of neural markers in plated neural cultures.

Cell-cycle and apoptosis analysis by flow cytometry

Cultured cells and embryoid bodies were incubated with bromodeoxyuridine, then, after adequate preparation of the cells, they were incubated with propidium-iodide, then flow cytometry was performed in order to analyze the distribution of the differentiating cells in cell-cycle and the rate of apoptosis.

Luciferase reporter assay

COS7 cells were cotransfected with Rybp cDNA construct and Plagl1 reporter construct, in various concentrations using $CaPO_4$ transfection. 48 hours after transfection the cells were lysed, then luciferase activity was recorded in a luminometer using reporter assay kit.

RESULTS

- *rybp*$^{-/-}$ embryoid stem cells could form embryoid bodies, but their ability to develop neural lineages was compromised. Morphological analysis showed that formation of "neural rosette-like" structures was accelerated in the *rybp*$^{-/-}$ embryoid bodies in comparison to the wild type. The *rybp*$^{-/-}$ derivatives grew less neurite processes and their organization into complex network was less extended in comparison to the wild type.
- the attenuation of pluripotency markers (Oct4, Nanog) was complete in both cell lines throughout *in vitro* neural differentiation.
- expression of polycomb repressive complex members (Ring1a, Ring1b, Ezh1, Ezh2) and their regulators (Yy1, Jmj), were slightly altered in the *rybp null* mutant in comparison to wild type.
- major germ layer (endoderm, mesoderm, ectoderm) formation was not impaired in *rybp null* mutant embryoid bodies.
- the expression of key early neural markers (e.g. Nestin, Pax6, NeuroD1) was accelerated in the *rybp null* mutant neural cultures compared to the wild type. Immunostaining showed that both the expression of early neural markers and the number of immunopositive cells were elevated in the mutant.
- key late neural markers (e.g. Tubb3, NeuN, Gfap, Olig2), axonal and dendritic markers (Tau, Map2) were downregulated in the mutant and the formation of axons and dendrites was defective as well.
- gene expression analysis showed that the induction of key neural transcription factor Plagl1 was deficient in the mutants during the entire course of neural differentiation.

- the distribution of cell-cycle phases was near identical in the wild type and *rybp*$^{-/-}$ cultures, suggesting that the absence of Rybp did not cause significant changes in cell-cycle regulation.
- the lack of Rybp caused elevated rate of apoptosis during neural differentiation.
- Rybp was able to activate the Plagl1 promoter.

CONCLUSION

In my thesis project I have investigated the role of Rybp in neural differentiation. For this purpose I utilized wild type and *rybp null* mutant embryonic stem cells and differentiated them *in vitro* into neural lineages. The *rbyp null* mutant stem cells were capable to form embryoid bodies and all primary germ layers. They were also able to develop neural stem cells, but they could not form matured neural cell types (neurons, astrocytes, oligodendrocytes). Our results showed that the absence of Rybp obscures terminal differentiation of neural lineages, since mutants cannot form mature neurons, astrocytes and oligodendrocytes properly from existing progenitors. We found that the endogenous Plagl1 is one of the most downregulated genes in the *rybp* mutants suggesting a possible transcription circuit between Rybp, Plagl1 and presumably other transcriptional factors. Our results demonstrated that Rybp is essential for the development of mature neural cell types.

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. ***Kovács, G.***, Szabó, V., Pirity, M.K., **Absence of Rybp Compromises Neural Differentiation of Embryonic Stem Cells.** *Stem Cells Int.* (2015)
doi: 10.1155/2016/4034620

(2.813 impact factor)

II. Ujhelly, O., Szabo, V., ***Kovacs, G.***, Vajda, F., Mallok, S., Prorok, J., Acsai, K., Hegedus, Z., Krebs, S., Dinnyes, A., Pirity, M.K. **Lack of Rybp in Mouse Embryonic Stem Cells Impairs Cardiac Differentiation.** *Stem Cells Dev.* 24(18):2193-205. (2015)
doi: 10.1089/scd.2014.0569

(3.727 impact factor)

Total impact factor: 6.54

Other, educational publication:

Kovacs, G. **Az élet forrása: Néhány tény az őssejtekről**
Élet és Tudomány 68:(34) pp. 1075-1077. (2013)