博士論文（要約）

Analyses of lysosomal functions during the differentiation process of neural stem/progenitor cells

（神経系前駆細胞の分化運命制御における
リソソームの新規機能の解明）

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Introduction

In the developing mouse telencephalon, neural stem-progenitor cells (NPCs) give rise to neurons and glial cells in a temporally and spatially regulated manner. The signaling pathways as well as transcriptional and posttranscriptional mechanisms that contribute to the maintenance of NPC and the onset of neuronal differentiation have been investigated extensively. However, relatively little is known about the regulation of NPC fate at the metabolic or organelle level.

Lysosomes, acidic organelles in the cell, fuse with autophagosomes and endosomes and play a role in cellular quality control by degrading proteins, nucleic acids, and lipids. In addition to degradation, lysosomes have also been shown to regulate the intracellular environment by sensing nutrients, storing metabolites, and acting as a hub for signaling molecules (Lim & Zoncu, 2016). Despite the wide range of functions of lysosomes, the state and specific roles of lysosomes in embryonic NPCs remain to be elucidated. In this study, I aimed to reveal the possible role of lysosomes in regulation of the maintenance versus the differentiation of NPCs.

Results

1. Lysosomes are more abundant in neural stem-progenitor cells than in differentiating neurons

I examined whether lysosomal amount is different between NPCs and differentiating neurons. NPCs ($CD133^+CD24^-$), immature neurons ($CD133^+CD24^+$), and neurons ($CD133^-CD24^+$) were defined by FACS from cells derived from the neocortex at embryonic day (E) 17.5 of mice, and acidified organelles including lysosomes were quantitatively assessed with the use of the fluorescent dye LysoTracker Red. The intensity of LysoTracker was higher in NPCs than in immature neurons and neurons. Quantitative PCR analysis showed that the expression of genes encoding lysosomal proteins such as lysosomal membrane protein LAMP1, protease cathepsin D, and lysosomal biogenesis regulators TFEB and TFE3 was higher in NPCs than in neurons (Fig. 1). In addition, immunohistochemical analysis at E13.5, E16.5, and postnatal day 0 showed that NPCs contained large granular LAMP1 signals. These results suggest that lysosomes are more abundant in NPCs than in neurons of the embryonic telencephalon.

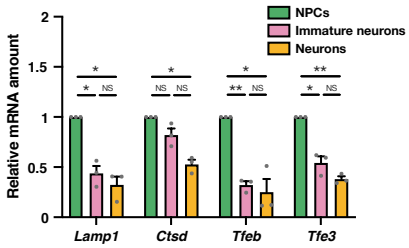

Figure 1 Lysosome-related genes were highly expressed in NPCs. (n=3, *p<0.05, **p<0.01)

2. Knockdown of TFEB and TFE3 promotes neurogenesis

To examine whether lysosome biogenesis is necessary for NPC maintenance, I knocked down TFEB and TFE3 by introducing constructs encoding corresponding shRNAs into neocortical NPCs at E14.5 by in utero electroporation. The fluorescence intensity of LysoTracker was confirmed to be decreased by knockdown of TFEB and TFE3 at E16.5. I found that knockdown of TFEB and TFE3 both reduced the fraction of cells positive for the NPC marker SOX2 or the intermediate progenitor cell (IPC) marker TBR2 and increased the fraction of cells positive for the immature neuron

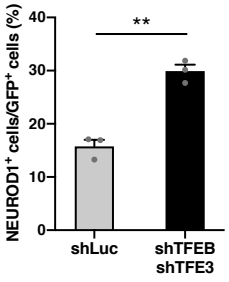

Figure 2 Knockdown of TFEB and TFE3 decreased the fraction of NEUROD1+ cells. (n=3, **p<0.01)

marker NEUROD1 in the Ncx at E17.5 (Fig. 2). These results suggested that the lysosomal biogenesis regulators TFEB and TFE3 are necessary for prevention of premature neurogenesis in neocortical NPCs.

3. Expression of an active form of TFEB suppresses neuronal differentiation

To further investigate whether the abundance of lysosomes contributes to the maintenance of NPCs, I examined the effects of forced expression of a constitutively active form of TFEB (TFEB-AA; Young et al., 2016) by in utero electroporation at E14.5. The expression of TFEB-AA indeed increased both the intensity of LysoTrakcer and the expression of lysosome-related genes. TFEB-AA expression markedly reduced the fraction of $TBR2^+$ cells at E16.5 (Fig. 3). Moreover, TFEB-AA expression increased the fraction of $SOX2^+$ cells at E17.5 (Fig. 4). These results together suggest that TFEB-AA suppresses neuronal fate commitment and maintains the undifferentiated state of NPCs.

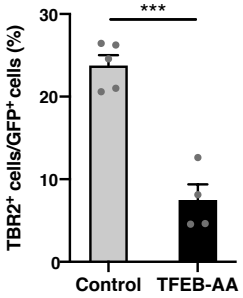

Figure 3 TFEB-AA expression decreased the fraction of $TBR2^+$ cells. (n=5 (Control), 4 (TFEB-AA), ***p<0.001)

4. The lysosomal transporter SLC15A4 is necessary for NPC maintenance

To investigate how TFEB might contribute the maintenance of NPCs, cells transfected with TFEB-AA were subjected to RNA sequencing and analysis of differentially expressed genes (DEGs). The number of DEGs was 296 in NPCs, whereas that in immature neurons was 2375. Interestingly, the expression of most NPC-enriched genes was upregulated by TFEB-AA. Among the lysosomal genes upregulated by TFEB-AA, I focused on SLC15A4, a lysosomal amino acids and short-peptide transporter, which is highly expressed in NPCs. Knockdown of SLC15A4 in neocortical NPCs by introduction of shRNAs at E14.5 resulted in a reduced fraction of $SOX2^+$ cells (Fig. 5). Thus, it was suggested that the TFEB target factor SLC15A4 plays an essential role in the maintenance of NPCs.

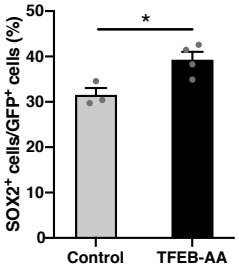

Figure 4 TFEB-AA expression increased the fraction of $SOX2^+$ cells. (n=3 (Control), 4 (TFEB-AA), *p<0.05)

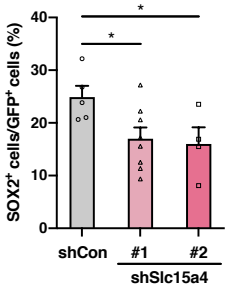

Figure 5 Knockdown of SLC15A4 decreased the fraction of $SOX2^+$ cells. (n=5 (Control), 8 (#1), 4 (#2), *p<0.05)

5. Lysosomes are abundant in embryonic origin of adult neural stem cells

Previous studies have revealed that most adult neural stem cells (NSCs) in the mouse subependymal zone (SEZ) are derived from a subpopulation of slowly dividing (or quiescent) embryonic NPCs (Furutachi et al., 2015; Fuentealba et al., 2015). Differences between rapidly dividing and slowly dividing NPCs in properties other than cell cycle frequency, particularly at the metabolic and organelle levels, have been largely unexplored. The results so far were consistent with the notion that abundant lysosomes (or a high level of TFEB) may contribute to the maintenance of the undifferentiated state of NPCs. I therefore examined whether slowly dividing embryonic NPCs contain an even greater number of lysosomes to support their long-term maintenance compared with other NPCs. To this end, expression of H2B-GFP was transiently induced in transgenic embryos with a Tet-on system at E9.5,

and NPCs ($CD133^{+}CD24^{-}$) that strongly retained H2B-GFP were collected as slowly dividing NPCs. I found that the fluorescence intensity of LysoTracker was significantly higher in H2B-GFP strongly positive NPCs than in other NPCs, suggesting that slowly dividing NPCs contain more lysosomes than do rapidly dividing NPCs. Furthermore, RT-qPCR analysis showed that the expression of lysosome-related genes such as those for LAMP1, cathepsins, and TFEB was tended to be greater in H2B-GFP strongly positive NPCs than in other NPCs (Fig. 6). These results suggested that lysosomal biogenesis is highly active in slowly dividing NPCs, including embryonic origin of adult NSCs. Given the results showing that lysosomes contribute to the maintenance of undifferentiated state of NPCs, it is indicated that lysosomal abundance may contribute to the long-term maintenance of slowly dividing NPCs until adulthood.

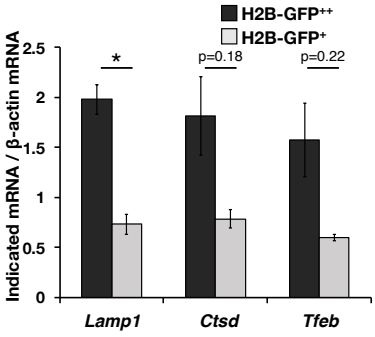

Figure 6 The expression of lysosome-related genes was higher in slowly dividing NPCs than in rapidly dividing NPCs. (n=3, *p<0.05)

Conclusion

I have here shown that lysosomes are more abundant in NPCs compared with differentiating neurons of the mouse embryonic telencephalon on the basis of LysoTracker Red fluorescence intensity and expression of lysosome-related genes as well as the accumulation of LAMP1-positive organelles in NPCs. The biogenesis of lysosomes may also be more active in NPCs relative to neurons, given the higher expression of genes for the lysosome biosynthesis regulators TFEB and TFE3 in $CD133^{+}CD24^{-}$ cells than in $CD133^{+}CD24^{+}$ cells and $CD133^{-}CD24^{+}$ cells. Knockdown of TFEB and TFE3 resulted in premature differentiation of neocortical NPCs, whereas, conversely, forced expression of a constitutively active form of TFEB (TFEB-AA) suppressed neuronal differentiation. I further showed that SLC15A4, a lysosomal amino acids and short-peptide transporter and downstream effector of TFEB, plays a role in the maintenance of NPCs. These lines of evidence point to a previously unrecognized function of lysosomes in the suppression of neuronal fate commitment and in maintenance of the undifferentiated state in NPCs of the developing mouse telencephalon.