Functions of a *jumonji–cyclin D1* pathway in the coordination of cell cycle exit and migration during neurogenesis in the mouse hindbrain

Miho Takahashi [a,b,1,2], Mizuyo Kojima [a,2], Kuniko Nakajima [a,2],
Rika Suzuki-Migishima [a], Takashi Takeuchi [a,b,*]

[a] *Mitsubishi Kagaku Institute of Life Sciences (MITILS), 11 Minamiooya, Machida, Tokyo 194-8511, Japan*
[b] *Graduate School of Environment and Information Sciences, Yokohama National University, 79-1 Tokiwadai, Hodogaya, Yokohama 240-8501, Japan*

Abstract

During development of the mouse central nervous system (CNS), most neural progenitor cells proliferate in the ventricular zone (VZ). In many regions of the CNS, neural progenitor cells give rise to postmitotic neurons that initiate neuronal differentiation and migrate out of the VZ to the mantle zone (MZ). Thereafter, they remain in a quiescent state. Here, we found many ectopic mitotic cells and cell clusters expressing neural progenitor or proneural marker genes in the MZ of the hindbrain of *jumonji* (*jmj*) mutant embryos. When we examined the expression of cyclin D1, which is repressed by *jmj* in the repression of cardiac myocyte proliferation, we found many ectopic clusters expressing both cyclin D1 and Musashi 1 in the MZ of mutant embryos. *jmj* is mainly expressed in the cyclin D1 negative region in the hindbrain, and cyclin D1 expression in the VZ was upregulated in *jmj* mutant mice. In *jmj* and *cyclin D1* double mutant mice, the ectopic mitosis and formation of the abnormal clusters in the MZ were rescued. These results suggest that a *jmj–cyclin D1* pathway is required for the precise coordination of cell cycle exit and migration during neurogenesis in the mouse hindbrain.
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Introduction

Regulation of cell cycle, differentiation and migration is critical for neurogenesis of the central nervous system. The proliferating neural progenitor cells are located in the ventricular zone (VZ), and self-renewal of these cells maintains the neural progenitor cell pool. After multiple rounds of cell proliferation, the neural progenitor cells exit the cell cycle and differentiate into neurons that migrate radially (radial migration) out of the VZ, and form the mantle zone (MZ). The timing of the onset of cell cycle exit of neural progenitor cells is closely related with the acquisition of distinct migratory behavior and identity of neuronal subtypes that are located at different positions with appropriate cell numbers. Therefore, coherent regulation of the cell cycle exit, migration and neuronal differentiation is essential for establishing brain cytoarchitecture with appropriate size and morphology.

Although the cell cycle exit, migration and neuronal differentiation appear to be regulated in a coordinated manner, the molecular mechanisms linking these events are not well understood. However, a number of molecules that play important roles in these events have been identified and their functions have been studied (for reviews, see Bertrand et al., 2002; Bielas et al., 2004; Ohnuma and Harris, 2003). In mice lacking the cyclin-dependent kinase inhibitors (CKIs) p19[INK4d] and p27[Kip1], many mitotic cells are scattered among postmitotic neurons in various parts of the adult brain, suggesting the uncoupling of cell cycle exit and migration (Zindy et al., 1999). Recent studies have shown that proneural basic helix–loop–helix (bHLH) transcription factors such as neurogenin 2 (Ngn2)

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and p27[Kip1] independently promote neuronal differentiation and radial migration in the cerebral cortex (Ge et al., 2006; Nguyen et al., 2006). p27[Kip1] stabilizes Ngn2, and p27[Kip1] and Ngn2 most likely form a regulatory loop that coordinates cell cycle exit with differentiation and the radial migration of cortical neurons (Nguyen et al., 2006).

The *jumonji* (*jmj*) gene and *jmj* mutant mice were originally obtained by a mouse gene trap strategy (Takeuchi et al., 1995). The trap vector was introduced into the *jmj* gene and disrupted the gene. *jmj* heterozygous mice (*jmj*[+/trap]) show no apparent abnormalities whereas *jmj* homozygous mice (*jmj*[trap/trap]), which lack *jmj* functions, die *in utero*. These embryos have various abnormalities that include neural tube, cardiac, and hematopoietic defects (for review, see Takeuchi et al., 2006). The *jmj* gene encodes a protein that is a member of both the AT-rich interaction domain (ARID) family and jumonji family. The ARID is a DNA-binding domain and several members of this family are known to be transcriptional factors and are involved in a variety of biological processes (Kortschak et al., 2000; Wilsker et al., 2005). All members of the jumonji family have a jmjC domain. Most recently, at least 7 human members have been shown to be histone demethylases and a jmjC is a catalytic domain of the enzymes (Cloos et al., 2006; Klose et al., 2006; Tsukada et al., 2006; Whetstine et al., 2006; Yamane et al., 2006). Although Jmj may not have histone demethylase activity because a jmjC domain lacks conserved residues for binding to co-factors, Jmj shows transcriptional repressor activity (Toyoda et al., 2003). Importantly, Jmj binds to the promoter of *cyclin D1*, a gene encoding one of the G1 cyclins, and represses the transcriptional activity. The repression is necessary for suppression of the cardiac myocyte proliferation (Toyoda et al., 2003). The lack of Jmj function results in the hyperproliferation of cardiac myocytes (Takeuchi et al., 1999; Toyoda et al., 2003).

In the present study, we found that a *jmj–cyclin D1* pathway is involved in cell cycle exit, migration, and neuronal differentiation in the hindbrain. We found many ectopic mitotic cells and cell clusters that express a proliferating neural marker, Musashi 1, in the MZ of the hindbrain of *jumonji* mutant embryos. Most of the clusters also expressed cyclin D1. Analysis of *jmj* and *cyclin D1* double mutant mice showed that loss of *cyclin D1* rescued the ectopic mitosis and formation of ectopic cell clusters expressing Musashi 1 in the MZ. The results suggest that a *jmj–cyclin D1* pathway is required for the proper cell cycle exit coupled with cell migration and neuronal differentiation in a spatially precise manner in the hindbrain.

Materials and methods

Mice

We used mice with a C3H/HeJ Jcl (Clea JAPAN, Inc., Tokyo, Japan) genetic background. The *jmj*[trap/trap] embryos were generated by intercrossing F26–32 *jmj*[+/trap] mice with C3H/HeJ mice. *jmj*[trap/trap] embryos that were viable and available for analyses were not obtained after E14.5 because of lethality.

cyclin D1 mutant mice (a FVB/NJ background, FVB.129S2(B6)–*Ccnd1*[tm1Wbg], original mice were reported by Sicinski et al., 1995) were

obtained from The Jackson Laboratory (Bar Harbor, ME). The heterozygous mice were backcrossed with wild C3H/HeJ mice twice and then, crossed once with *jmj*[+/trap] mice to produce double heterozygous mice. Double homozygous mice were obtained by crossing with these double heterozygous mice.

Mice were genotyped by PCR as described previously (Sicinski et al., 1995; Takeuchi et al., 1995). The presence of a vaginal plug was regarded as E0.5.

Experiments involving animals were performed in accordance with standard ethical guidelines for the care and use of laboratory animals in National Institute of Health (1985) and were approved by ethical committee in our institute.

Histology, immunostaining and counting

Embryos were dissected in Dulbecco's phosphate buffered saline (PBS) and then fixed overnight at 4°C in Bouin's fixative (Sigma Diagnostics, St. Louis, MO) or for 3 h at room temperature in Streck Tissue fixation (Streck Lab.) for hematoxylin only or hematoxylin and eosin (HE) staining or immunostaining. Other embryos were fixed overnight in 4% paraformaldehyde in PBS for immunostaining, or for 30 min to 2 h in 0.2% glutaraldehyde–1% formaldehyde–0.02% NP40 in PBS for X-gal staining. Embryos were sectioned transversely. HE staining and X-gal staining were performed as described previously (Takeuchi et al., 1999). Cells which showed chromosomes but neither nuclear membrane nor nucleoli were counted as mitotic cells in sections stained with hematoxylin or HE. For immunostaining, the following primary antibodies were used: neural class III β tubulin (mouse IgG, TuJ1, Covance; rabbit IgG, a gift from Dr. Arimatsu), phosphorylated histone H3 (rabbit IgG, Upstate Biotechnology), BrdU (mouse IgG, Bu20, DAKO), Musashi 1 (rat IgG, a gift from Dr. Okano), cyclin D1 (mouse IgG, 72-13G, Santa Cruz; rabbit monoclonal, RM-9104, LAB VISION), Mash 1 (mouse IgG, 556604, BD Pharmingen), Sox 2 (goat IgG, Y-17, Santa Cruz), PECAM 1 (goat IgG, M-20, Santa Cruz) and phosphorylated vimentin (mouse IgG, 4A4, MBL).

Mitotic cells recognized with hematoxylin staining or positive cells with antibodies were counted in 5–10 sections per mouse and 2–6 mice were analyzed.

BrdU (0.2 mg/body weight, 10 mg/ml PBS, Amersham Pharmacia Biotech) was injected intraperitoneally 2 h before sacrifice of the mice.

MetaMorph software (Molecular Devices) was used for quantification of the intensity of fluorescence.

Statistical analysis

The experimental data were analyzed using Student's *t* test. Fisher's least significant difference (LSD) test was used after obtaining a significant difference with one-way analysis of variance (ANOVA) for multiple comparison tests (Fig. 8B). Values of $P < 0.05$ were considered statistically significant.

Results

Ectopic mitotic cells in the mantle zone of the hindbrain in jumonji mutant embryos

Because almost all *jumonji* (*jmj*) homozygous (*jmj*[trap/trap]) embryos with a C3H/HeJ background have been shown to die around embryonic day 11.5 (E11.5) due to cardiac defects (Takahashi et al., 2004), it is difficult to analyze the functions of *jmj* in the central nervous system after middle embryonic stages using mutant mice. However, we found that a minor portion of *jmj*[trap/trap] embryos survive until E13.5. These embryos show neural tube defects (NTD) (Fig. 1Ac). We examined histologically the brain of these surviving *jmj* mutant embryos at E13.5 to determine the functions of *jmj* in the central nervous system at the middle embryonic stage.

When we examined cross sections of these embryos, several morphological abnormalities were found. The gross