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Altered cerebellum development and impaired motor coordination in mice lacking the Btg1 gene: Involvement of cyclin D1

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ABSTRACT

Cerebellar granule neurons develop postnatally from cerebellar granule precursors (GCPs), which are located in the external granule layer (EGL) where they massively proliferate. Thereafter, GCPs become postmitotic, migrate inward to form the internal granule layer (IGL), further differentiate and form synapses with Purkinje cell dendrites.

We previously showed that the Btg family gene, Tis21/Btg2, is required for normal GCP migration. Here we investigated the role in cerebellar development of the related gene, Btg1, which regulates stem cell quiescence in adult neurogenic niches, and is expressed in the cerebellum.

Knockout of Btg1 in mice caused a major increase of the proliferation of the GCPs in the EGL, whose thickness increased, remaining hyperplastic even after postnatal day 14, when the EGL is normally reduced to a few GCP layers. This was accompanied by a slight decrease of differentiation and migration of the GCPs and increase of apoptosis. The GCPs of double Btg1/Tis21-null mice presented combined major defects of proliferation and migration outside the EGL, indicating that each gene plays unique and crucial roles in cerebellar development. Remarkably, these developmental defects lead to a permanent increase of the adult cerebellar volume in Btg1-null and double mutant mice, and to impairment in all mutants, including Tis21-null, of the cerebellum-dependent motor coordination. Gain- and loss-of-function strategies in a GCP cell line revealed that Btg1 regulates the proliferation of GCPs selectively through cyclin D1. Thus, Btg1 plays a critical role for cerebellar maturation and function.

1. Introduction

The cerebellum is required for motor coordination and learning, and is implicated in cognition and emotion as well as in related pathologies such as autism (Reeber et al., 2013). Thus, elucidating the mechanisms controlling the generation of the cerebellar neurons is critical to understand these processes and their pathologies.

During mid gestation, a subset of progenitor cells in the brain ventricular zone migrates to the rhombic lip, germinative epithelium located at the roofplate of the fourth ventricle, where they are specified to the neural lineage under the influence of bone morphogenetic proteins and of the transcription factor Math1 (Alder et al., 1996, 1999; Ben-Arie et al., 1997; Wang and Wechsler-Reya, 2014). These progenitors migrate from the upper rhombic lip over the surface of the cerebellar anlage to form the external granule layer (EGL) of cerebellar primordia, where they become precursor cells of the cerebellar granule neurons (GCPs; Wingate, 2001). In the EGL the GCPs continue to proliferate actively, in the mouse until the second postnatal week, under the influence of Sonic Hedgehog, secreted by the Purkinje cells (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999).

Finally, GCPs exit the cell cycle and migrate inward to the cerebellar internal granule layer (IGL) below the Purkinje cell soma, differentiating into mature granule neurons (Fujita et al., 1966; Rakic, 1971). The signals triggering these processes are poorly

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understood. They play a key role not only in the normal development of the cerebellum, but also, when a misregulation occurs, in the formation of medulloblastoma, a most common childhood tumor arising in 20% of cases from GCPs (Kadin et al., 1970; Marino, 2005; Schüller et al., 2008; Yang et al., 2008; Gibson et al., 2010).

In this context, we have recently provided evidence for the role played as a medulloblastoma suppressor by the antiproliferative and prodifferentiative gene Tis21/Btg2 (also known as PC3, hereafter referred to as Tis21; Bradbury et al., 1991; Rouault et al., 1996). Overexpression of this gene inhibits the proliferation of normal and neoplastic GCPs and facilitates their differentiation (Farioli-Vecchioli et al., 2007). Surprisingly, however, when Tis21 is ablated, in cerebellum the proliferation of GCPs does not change, but their migration is strongly inhibited in consequence of the decrease of the chemokine Cxcl3 within the GCPs. This defect of migration causes a longer exposure of GCPs to Shh and a great increase of medulloblastoma frequency (Farioli-Vecchioli et al., 2012a,b).

Given the antiproliferative action of Tis21 in several neural and non-neural tissues (Boiko et al., 2006; Evangelisti et al., 2009; Montagnoli et al., 1996; Farioli-Vecchioli et al., 2009, 2014a), a question arising from these findings is whether Tis21-null GCPs do not increase their proliferative rate because other genes substitute for the absence of Tis21. Among these may be the Tis21 family-related gene Btg1, which is expressed in several neural tissues including cerebellum, and also in the subventricular zone and dentate gyrus, where it inhibits the proliferation of adult neural stem cells and controls their self-renewal (Farioli-Vecchioli et al., 2012c). It is known that Btg1 inhibits cellular proliferation (Rouault et al., 1992; Li et al., 2009; Zhu et al., 2013) by regulating the S-phase of the cell cycle (Farioli-Vecchioli et al., 2014b).

We therefore analyzed the functional role of Btg1 in the proliferation, differentiation and migration of GCPs and its role in cell cycle control. We found that Btg1 is required to negatively control the proliferation of GCPs and, to a lesser extent, their differentiation; furthermore, Btg1 controls the G1 to S phase transition by quite selectively regulating cyclin D1 expression. By using double mutant Btg1/Tis21-null mice we revealed the specific requirement of Btg1 to control the proliferation of the GCPs, whereas their migration was essentially dependent on Tis21. Furthermore, the developmental defects arising from the ablation of Btg1 alone or with Tis21 result in permanent deficits of motor coordination, thus highlighting the importance of Btg1 in the normal development and function of the cerebellum.

2. Materials and methods

2.1. Mouse lines and genotyping

The Btg1 knockout mouse line was previously generated in the C57BL/6 strain as described (Farioli-Vecchioli et al., 2012c), by inserting the neomycin resistance cassette within exon I of the Btg1 gene. Genotyping of mice was routinely performed by PCR analysis, using genomic DNA from tail tips as described (Farioli-Vecchioli et al., 2012c). The Tis21 knockout mouse line was previously generated and described (Farioli-Vecchioli et al., 2009). The double mutant $Btg1^{-/-}$/$Tis21^{-/-}$ mice and the $Btg1^{+/+}$/$Tis21^{+/+}$, $Btg1^{-/-}$/$Tis21^{+/+}$, $Btg1^{+/+}$/$Tis21^{-/-}$ mice (referred throughout this report to as wild-type, $Btg1^{-/-}$ and $Tis21^{-/-}$, respectively) were generated by multiple intercrossing between $Btg1^{-/-}$ and $Tis21^{-/-}$ mice, until an isogenic progeny was obtained. Math1-green fluorescent protein mice (Math1-GFP) express GFP driven by the Math1 enhancer (Lumpkin et al., 2003), and were crossed to the $Btg1^{-/-}$ mice to obtain GFP/$Btg1^{-/-}$ mice. Cerebellar morphology and volumes were measured in male mice; the other experiments were performed with mice of both sexes, and all animal procedures were completed in accordance with the current European (directive 2010/63/EU) Ethical Committee guidelines. Btg1 knockout mice are available upon request to J.-P. Rouault.

2.2. Bromodeoxyuridine treatment of mice and cell lines

GCPs entering S-phase were detected 1 h after an injection of bromodeoxyuridine (BrdU) (95 mg/ kg, i.p.), according to existing protocols (Canzoniere et al., 2004; Qiu et al., 2010).

GCPs migrating from the EGL to the inner layers were visualized either 42 h or 5 days after a single injection of BrdU (95 mg/ kg, i.p.) in P7 (postnatal day 7) and P14 mice.

BrdU incorporation in C17.2 cerebellar cells, in cyclin $D1^{+/+}$ and cyclin $D1^{-/-}$ mouse embryo fibroblasts (MEFs) and in DAOY medulloblastoma cells, was performed as previously described (Guardavaccaro et al., 2000), by adding 50 μM BrdU to exponentially growing cultures 18 h (or 24 h in MEFs) before fixation.

2.3. Immunohistochemistry: sample preparation, BrdU labeling, antibodies, and image analysis

Immunohistochemistry was performed on free-floating cerebellar sections, stained for multiple labeling and BrdU incorporation by fluorescent methods, as described (Canzoniere et al., 2004; Farioli-Vecchioli et al., 2007). Briefly, for EGL analysis, cerebella of P7 and P14 mice were dissected and fixed by overnight immersion in 4% PFA in PBS; cerebella of P60 mice were dissected after transcardiac perfusion with 4% PFA in PBS and kept overnight in PFA. Fixed cerebella were then equilibrated in 30% sucrose in PBS and cryopreserved at −80 °C until use. Cerebella were then embedded in Tissue-Tek OCT (Sakura Finetek, CA, USA) and cut at −25 °C in midsagittal sections of 40 μm. BrdU-labeled or cyclin-positive cells were detected in sections pretreated to denature the DNA, with 2 N HCl 45 min at 37 °C and then with 0.1 M sodium borate buffer, pH 8.5, for 10 min. The following primary antibodies were used: a rat monoclonal antibody against BrdU (AbD Serotec; MCA2060; 1:300), goat polyclonal antibodies against NeuroD1 (R&D Systems; AF2746; 1:100) or Calbindin (Santa Cruz Biotechnology, CA, USA; sc-7691; 1:100), a mouse monoclonal antibody raised against NeuN (Merck Millipore, MA, USA; MAB377; 1:100), or rabbit polyclonal antibodies against cleaved (activated) Caspase-3 (Cell Signaling Technology; 9661; 1:100) or GFAP (DakoCytomation, Denmark; Z0334; 1:250). Moreover, cyclins were detected in the EGL sections by using a rabbit monoclonal antibody against cyclin D1 (Merck Millipore; clone EP272Y; 1:50), or rabbit polyclonal antibodies against cyclin D2 (Santa Cruz; sc-593; 1:50) or cyclin A (Santa Cruz; sc-596; 1:50).

Images of the immunostained sections were captured by a confocal laser scanning TCS SP5 microscope (Leica Microsystems) and were analyzed by the I.A.S. software (Delta Sistemi, Rome, Italy).

2.4. Cell culture and cell lines

C17.2 cells-an immortalized line of cerebellar precursor cells (Ryder et al., 1990)-and cyclin $D1^{+/+}$ and cyclin $D1^{-/-}$ MEFs were cultured in DMEM (Guardavaccaro et al., 2000), while DAOY cells in Minimum Essential Medium (MEM), containing 10% fetal calf serum. All cell lines were kept in a humidified atmosphere of 5% CO_2 at 37 °C.