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WLS EXPRESSION IN THE RHOMBIC LIP ORCHESTRATES THE EMBRYONIC DEVELOPMENT OF THE MOUSE CEREBELLUM

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Abstract—Wntless (Wls) is implicated in the Wnt signaling pathway by regulating the secretion of Wnt molecules. During brain development, Wls is expressed in the isthmic organizer (ISO) and rhombic lip (RL). Wls regulates Wnt1 secretion at the ISO which is required to induce midbrain–hindbrain structures. However, Wls function in the RL is not known. Here, we employed *Nestin*-cre to delete *Wls* specifically in the RL during mid-gestation. The loss-of-*Wls* leads to an abnormal RL during development and cerebellar vermis hypoplasia at birth. The *Wls* conditional knockout (cKO) has rudimentary foliation with an absence of Bergmann glia fibers in the external germinal layer (EGL). The *Wls*-cKO cerebellum also exhibits ectopia of several cell types and aberrations in granule cell organization. Finally, there is a loss of 85% of unipolar brush cells. From these findings, Wls-expressing cells in the rhombic lip are implicated in the orchestration of cerebellar development.

Key words: Wntless, cerebellar development, rhombic lip, cerebellar hypoplasia, neuronal ectopias, granule cells.

INTRODUCTION

Wls is a transmembrane protein that controls the secretion of Wnt molecules (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). During early embryogenesis, Wls is expressed at the isthmic organizer and mediates the secretion of Wnt1 (Fu et al., 2011), which patterns the neural tube and induces the development of cerebellar anlage from rhombomere 1 (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Deletion of *Wls* or *Wnt1* from the ISO results in the loss of cerebellar structure (Thomas and Capecchi, 1990; McMahon and Bradley, 1990; Carpenter et al., 2010; Fu et al., 2011). Recently, we found that later in cerebellar development, Wls expression is localized to the interior face of the rhombic lip (iRL) and aids in parcelling the RL into four molecularly distinct compartments (Yeung et al., 2014). Furthermore, Wls expression is restricted to the iRL by the presence of Pax6 in the complementary exterior face of the RL (eRL) and external germinal layer (EGL), such that in the *Pax6*-null small eye (*Sey*) mutant, Wls expression expands into the eRL and EGL (Yeung et al., 2014). However, the requirement of Wls in the RL and how it interacts with Pax6 expression is unclear.

There are two challenges in studying *Wls* in cerebellar development: 1) the early embryonic lethality of the conventional *Wls* knockout (Yeung et al., 2014), due to the defect in primitive streak and mesoderm formation (Fu et al., 2009), and 2) when using a conditional knockout (cKO) one needs to choose a Cre-line that does not affect the ISO, as it is known that in a *Wnt1-Cre* cKO the cerebellar anlage never develops attesting to the critical involvement of the ISO in cerebellar development (Carpenter et al., 2010; Fu et al., 2011). One means to bypass both of these constraints in studying the role of *Wls* in RL and cerebellar development, is to create a cKO that has a later onset of deletion to permit further development and to dissociate the use of *Wls* in the ISO from the RL.

To circumvent the early requirement of *Wls*, we employed a *Nestin*-cre line to inactivate *Wls* in the cerebellum at mid-gestation. With this approach, we deleted Wls expression from the RL at E12.5 while the earlier expression at the ISO remains intact in the *Wls*-cKO. The *Wls*-cKO mutants die soon after birth and examination of the cerebellum at P0 reveals a profound reduction in the size of the vermis with rudimentary foliation. The cerebellum of the *Wls*-cKO exhibits several cellular abnormalities, including: a) A smaller RL, b) diminished nuclear and cytoplasmic β-catenin in cells of the RL, c) ectopic clusters of granule cells in the lateral cerebellar core, d) EGL that has an uneven thickness and contains gaps devoid of granule cells, e) ectopic Purkinje cells (PCs) in the EGL gaps and the inferior colliculus, f) ectopic interneurons in the EGL gaps and the pial surface, g) an absence of Bergman glia fibers in the EGL, and h) a significant reduction in the number of unipolar brush cells (UBCs). Thus, our

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Abbreviations: BGC, Bergmann glia cell; cKO, conditional knockout; CN, cerebellar nuclear; CP, choroid plexus; E, embryonic; EGL, external germinal layer; eRL, exterior face of the rhombic lip; GCP, granule cell progenitor; GC, granule cell; IHC, immunohistochemical; iRL, interior face of the rhombic lip; ISO, isthmic organizer; MB, midbrain; NTZ, nuclear transitory zone; P, postnatal; PCP, planar cell polarity; PCs, Purkinje cells; RL, rhombic lip; Sey, small eye (mutant); UBCs, unipolar brush cells; Wls, Wntless.

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present findings illustrate crucial roles of Wls in the developing cerebellum.

EXPERIMENTAL PROCEDURES

Mouse strains and husbandry

The *Nestin*-cre mouse strain (B6.Cg-Tg(Nes-cre)1Kln/J), *Nes-Cre*, was obtained from Jackson Laboratory, Bar Harbor, ME (Jax #003771). Animals were genotyped by standard PCR according to the protocol described by the Jackson Laboratory.

The floxed *Wls* mouse strain, Wls^{flox}, was a gift from Richard Lang, University of Cincinnati, and is available from Jackson Laboratory, Bar Harbor, ME (Jax # 012888). Animals were genotyped by standard PCR according to protocol previously described (Carpenter et al., 2010).

We previously generated the *Wls*-null allele mouse strain, Wls^{LacZ}. The generation, breeding and genotyping protocols of this strain are described previously in detail (Yeung et al., 2014).

Experimental *Wls*-cKO (*Nes-Cre*/+, $Wls^{LacZ/flox}$) mice were generated by the following mating schema: 1) Hemizygous *Nes-Cre*/+ mice were mated with heterozygous $Wls^{LacZ/+}$ mice to generate the double heterozygous *Nes-Cre*/+, Wls^{LacZ}/+ mice. 2) The double heterozygous *Nes-Cre*/+, Wls^{LacZ}/+ mice were mated to homozygous $Wls^{flox/flox}$ to generate the experimental control (+/+, Wls^{flox}/+) and cKO mice.

The morning a vaginal plug was detected was designated as embryonic day 0.5 (E0.5). All studies were conducted in accord to the protocols approved by Institutional Animal Care and Use Committee and Canadian Council on Animal Care at the University of British Columbia.

Tissue preparation and histology

Tissues were collected at each day from E11.5 to P0. Embryos harvested between E11.5 to E15.5 were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 1 h at 4 °C. Embryos and animals harvested at E16.5 or later were perfused with 4% paraformaldehyde in 0.1 M PB, the brain isolated and further fixed in 4% paraformaldehyde in 0.1 M PB for 1 h at room temperature. Fixed tissue was rinsed with PBS, followed by cryoprotection with 30% sucrose/PBS overnight at 4 °C before embedding in OCT compound. Tissues were sectioned at 12 μm for immunohistochemistry and cryosections were mounted on Superfrost™ slides (Fisher), air dried at room temperature, and stored at −80 °C until used. In all cases, observations were based on a minimum of 3 brains per genotype per experiment.

Immunohistochemistry

Tissue sections were rehydrated in PBS. For brightfield immunohistochemistry endogenous peroxidase activity was inhibited by treating the sections with 1% H2O2 in PBS followed by PBS-T (0.1 M PBS/0.1% Triton X-100) rinse. Sections were incubated at room temperature for 20 min with blocking solution (1% BSA and 5% normal serum in PBS-T) and subsequently incubated at room temperature overnight with primary antibodies in a humid chamber. Following PBS-T washes the sections were incubated with biotinylated secondary antibodies (at 1:200, Vector Laboratories, Burlingame, CA) and processed for PAP immunohistochemistry using the ABC Kit (Vector Laboratories) according to the manufacturer's protocol. Slides were dehydrated and coverslips were applied. For immunofluorescence, secondary antibodies labeled with fluorochrome were used to recognize the primary antibodies. The slides were coverslipped with FluorSave (Calbiochem, 345789). Primary antibodies used were as follows: rabbit anti-ATOH1 (1:500, proteintech, 21215-1-AP), chicken anti-β-GAL (1:10,000; Abcam, Ab9361), rat anti-BrdU (1:300, Abcam, Ab6326), rabbit anti-CALBINDIN (1:1000, Millipore, AB1778), rabbit anti-active CASPASE-3 (1:500; Abcam, Ab13847), guinea pig anti-GLAST (1:1000, Millipore, AB1782), rabbit anti-non-phospho β-CATENIN (1:1000, Cell Signaling Technology, 19807), mouse anti-NEUN (1:200, Millipore, MAB377), mouse anti-NESTIN (1:100, Millipore, MAB353), rabbit anti-PAX2 (1:200, Invitrogen, 71-6000), rabbit anti-PAX6 (1:200; Covance, PRB-278P), rabbit anti-TBR1 (1:800; Abcam, Ab31940), rabbit anti-TBR2 (1:800; Millipore, AB2283), rabbit anti-WLS (1:1000, Seven Hills Bioreagents, WLAB-177).

Cell counts and areal analysis

We estimated the numbers of CN neuron, UBCs and dying cells by counting cells positive for the appropriate cell marker (Tbr1 for CN neurons, Tbr2 for UBCs, caspase-3 for apoptotic cells). Cerebellar area and EGL area measures were taken from every 10th section across the half P0 cerebellum with the aid of the Axiocam/Axiovision hardware–software components (Carl Zeiss). In all cases, final numbers were based on a minimum of 3 cerebella per genotype. Statistical significance between control and cKO was determined by a two-tailed student's *t*-test.

Microscopy

Imaging for analysis and photomicroscopy were performed using a Zeiss Axiovert 200 M microscope with the Axiocam/Axiovision hardware–software components (Carl Zeiss). Confocal microscopy was performed using a Leica SP8X confocal microscope, and captured using the Leica application suite (LAS X 2.0.1.14392).

RESULTS

Wls is inactivated in the RL during mid-gestation by *Nestin-Cre*-mediated recombination

To conditionally inactivate *Wls* in the RL at mid-gestation, we employed the *Nestin-Cre* line (Tronche et al., 1999) that has been characterized to drive Cre expression in nervous tissues starting at E10.5 (Graus-Porta et al., 2001). In the cerebellar RL, Nestin expression is found