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Oncogenic *Lmo3* cooperates with *Hen2* to induce hydrocephalus in mice

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Abstract: We previously reported that LMO3 and HEN2 act as oncogenes in neuroblastoma development through up-regulating *MASH1* transcription by interfering with HES1. To confirm these results *in vivo*, we generated transgenic mice of these genes. *Lmo3* or *Hen2* was expressed under the control of *Wnt1* promoter, which is expressed in the central nervous system and neural crest of the sympathoadrenal lineage from which neuroblastoma develops. Heterozygous *Lmo3* and *Hen2* transgenic mice (*Tg (Lmo3)* and *Tg (Hen2)*) developed hydrocephalus at higher frequency than for the wild type mice, and all heterozygous double-transgenic mice (*Tg (Lmo3*; *Hen2)*) developed hydrocephalus. Therefore, *Lmo3* and *Hen2* may be involved in and have synergistic effects on hydrocephalus development. Although aqueduct stenosis occurred in all genotypes, it was mild in *Tg (Lmo3*; *Hen2)* mice. Furthermore, hydrocephalus was detected at E18.5 in *Tg (Lmo3*; *Hen2)*. These results suggest that the causes of hydrocephalus are not only aqueduct stenosis but also disorder of neocortical development. A similar phenotype was reported in *Robo1/2*$^{-/-}$ mice, in which *Hes1* expression level was decreased in ventricular zone progenitors. Thus, it is suggested that the expression levels of *Lmo3* and/or *Hen2* could determine the fate of stem cells by inhibiting *Hes1* function during nervous system development and might be a trigger of aberrant neurogenesis *in vivo*.
Key words: hydrocephalus, neuroblastoma, neuronal development

Introduction

Neuroblastoma is one of the typical childhood cancers and originates from the sympathetic cell lineage of the neural crest [10, 11]. The LMO (LIM domain only) protein family is composed of four members, LMO1, LMO2, LMO3 and LMO4. Although LMO proteins lack DNA-binding activity, accumulating evidence suggests that they are involved in the transcriptional regulation of specific target genes in collaboration with other tran-

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scription factors [2]. Genetic analyses demonstrated that LMO1 and LMO2 contribute to the genesis of immature and aggressive T-cell leukemia [12], whereas LMO4 was implicated in the development of breast cancer [13, 14]. Previously, we reported that *LMO3* is expressed at significantly high levels in human unfavorable neuroblastomas relative to favorable ones, and has oncogenic potential in neuroblastoma [1]. LMO3 formed a complex with neuronal-specific basic helix-loop-helix (bHLH) transcription factor HEN2 (Helix-Loop-Helix protein 2), which was also expressed at higher levels in unfavorable neuroblastoma than in the favorable type, raising the possibility that LMO3 may form a complex with HEN2 and play an important role in the genesis and development of neuroblastoma through transcriptional regulation of as-yet-unidentified target gene (s).

A proneural bHLH transcription factor termed MASH1 (Mammalian Achaete Scute Homolog 1) plays a critical role in the development of sympathetic neurons and is highly expressed in neuroblastoma [6, 7]. A bHLH protein termed HES1 (Hairy and Enhancer of Split 1) acts as a negative regulator for MASH1 [9]. We have already reported that there could be a functional relationship between LMO3/HEN2 and MASH1 in neuroblastoma, and found that LMO3/HEN2 attenuates HES1 function and enhances the transactivation of *MASH1*, leading to an aggressive phenotype of neuroblastoma [8].

In this study, transgenic mice of *Lmo3* and *Hen2* were established in order to study their roles in the development and tumorigenesis of the nervous system using promoter of *Wnt1*, which is expressed in neural crest cells and the central nervous system. It was suggested that oncogenic *Lmo3* could cooperate with *Hen2* to induce aberrant neurogenesis, hydrocephalus, in mice.

Materials and Methods

Mice

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology of Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of Chiba Cancer Center (Permit Number:14–14). All efforts were made to minimize suffering. All mice had the C57BL/6J background.

DNA constructs

To generate pWEXP-3C-HA-*Lmo3* or pWEXP-3C-FLAG-*Hen2*, HA-*Lmo3* or FLAG-*Hen2* transgene, the full-length cDNA was amplified from mouse brain cDNA library by PCR and cloned into the *Wnt1* expression vector pWEXP-3C [4]. The transgenes were digested with restriction endonuclease *Sal*I before microinjection.

Production and genotyping of transgenic mice

C57BL/6J-*Tg (Wnt1-HA-Lmo3)* (*Tg (Lmo3)*) and C57BL/6J-*Tg (Wnt1-FLAG-Hen2)* (*Tg (Hen2)*) mice were generated by the microinjection of linear DNA fragments into pronuclei of BDF_1 (C57BL/6J × DBA2J) zygotes. Genotyping of transgenic mice was carried out by PCR using rTaq (Takara) under the following conditions: 95°C 5 min (1 cycle); 95°C 20 s, 58°C 20 s, 72°C 45 s (25 cycles) (FLAGF/Hen2R), or 95°C 5 min (1 cycle); 95°C 20 s, 66.5°C 20 s, 72°C 45 s (25 cycles) (HAF/Lmo3R). Primers used for the analysis were as follows: FLAGF, 5′-ATGGACTACAAGGACGACG-3′; Hen2R, 5′-TTGAAAGCCTCCACTCGGATG-3′; HAF, 5′-ACCCATACGATGTTCCGGATTACGC-3′; and Lmo3R, 5′-GGATCCTCAGCGGACCTGGGGTGC-3′.

Reverse transcription-PCR analysis

Total RNA was prepared from tissues by using the RNeasy Mini Kit (Qiagen) and NucleoSpin totalRNA FFPE (Machery-Nagel) following the manufacturer's protocol. Reverse transcription was carried out using random primers and SuperScript II (Invitrogen) and iScript cDNA Synthesis Kit (Bio-Rad). Following the reverse transcription, the resultant cDNA was subjected to PCR-based amplification. The cDNA was amplified for 95°C 5 min (1 cycle); 95°C 20 s, 58°C 20 s, 72°C 45 s (40 cycles) (FLAGF/Hen2R), 95°C 5 min (1 cycle); 95°C 20 s, 50°C 20 s, 72°C 45 s (40 cycles) (HAF/Lmo3R2), 95°C 5 min (1 cycle); 95°C 20 s, 56°C 20 s, 72°C 45 s (35 cycles) (Mash1F/R), 95°C 5 min (1 cycle); 95°C 20 s, 60°C 20 s, 72°C 45 s (35 cycles) (Hes1F2/Hes1R2), 95°C 5 min (1 cycle); 95°C 30 s, 50°C 30 s, 72°C 30 s (40 cycles) (Actb (actin, beta) F2/R2), 95°C 2 min (1 cycle); 95°C 30 s, 60°C 2 min (30 cycles) (Gapdh (glyceraldehyde-3-phosphatede hydrogenase) F/R). RT-PCR Primers used for the analysis were as follows: Lmo3R2, 5′-CTGATTGCAAAGCTGACAGG-3′; Hes1F2, 5′-ACACCGGACAAACCAAAGAC −3′; Hes1R2, 5′-TGTCTGCCTTCTCTAGCTTGG −3′; Mash1F, 5′-GTTGGTCAACCTGGGCTTT-3′; Mash1R, 5′-GCCATGGAGTTCAAGTCGTT-3′; Gapdh F,

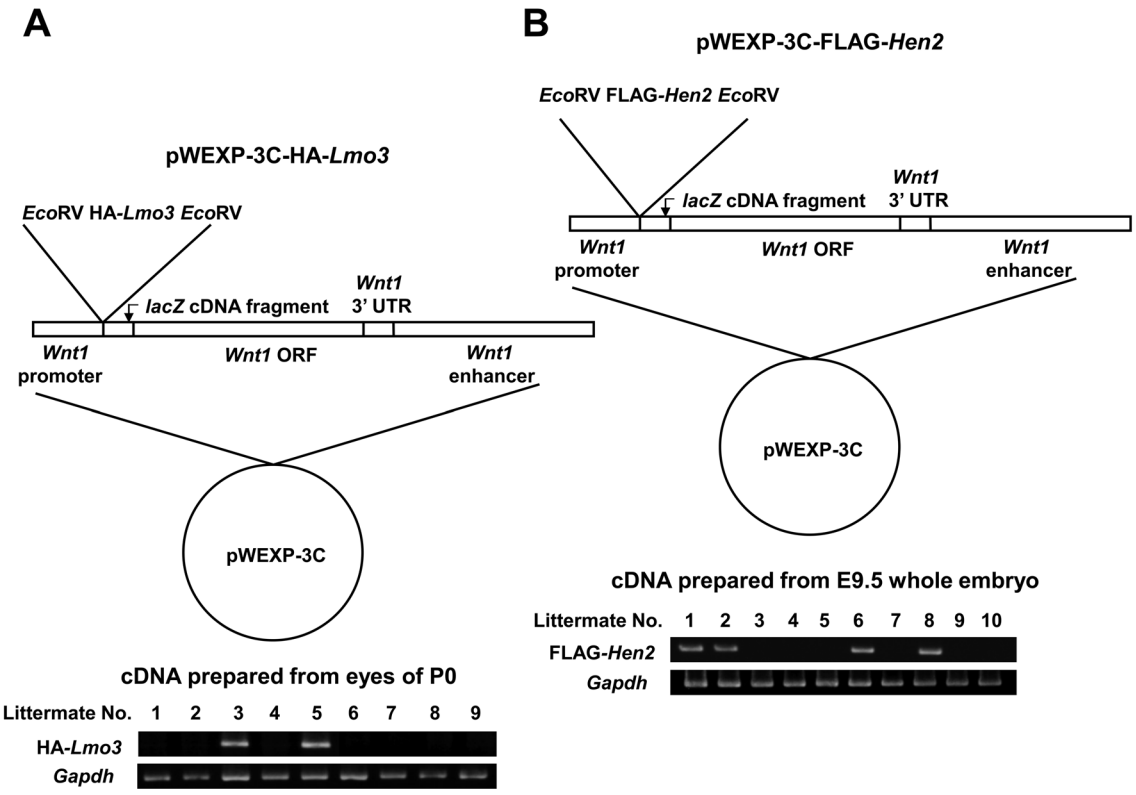

Fig. 1. Constructs in which transgenes HA-*Lmo3* and FLAG-*Hen2* are expressed under the control of *Wnt1* promoter. Plasmids pWEXP-3C-HA-*Lmo3* and pWEXP-3C-FLAG-*Hen2* comprise HA-tagged full-length *Lmo3*(A) and FLAG-tagged full-length *Hen2*(B) cloned into the pWEXP-3C expression vector (13) in order to the express transgenes under the control of *Wnt1* promoter. Expression of transgenes in mice was confirmed by RT-PCR using tissue cDNA prepared from the eyes of P0 pups or E9.5 whole embryos (A and B).

5′-ATCTTCTTGTGCAGTGCCAG-3′; GapdhR, 5′-ATCTTCTTGTGCAGTGCCAG-3′; ActbF2, 5′-ACCTCATGAAGATCCTGACC −3′; and ActbR2, 5′-CGTTGCCAATAGTGATGACC-3′. The products were subjected to agarose gel electrophoresis. cDNA integrity was confirmed using *Actb* or *Gapdh*.

Immunohistochemistry and morphometry

The brains were fixed in 4% paraformaldehyde at 4°C overnight. Dehydrated samples were embedded in paraffin and sectioned, which were stained with hematoxylin and eosin. Specimens were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. The following primary antibodies were used: mouse anti-NEUN (1: 500, Millipore, MAB377), mouse anti-GFAP (1: 500, SIGMA, G3893), rabbit anti-OLIG2 (1: 200, IBL, 18953), rabbit anti-IBA1 (1: 500, Wako, 019–19741). The numerical density of immunoreactive cells was counted by setting the ROI in the periaqueductal gray matter.

Statistical analysis

Statistical significance was calculated by 2 × 2 Chi square test (Fisher's test). A P-value<0.05 was considered statistically significant and a P-value<0.01 was considered highly statistically significant.

Results

Transgenes are expressed under the control of Wnt1 promoter in transgenic mice

In order to express *Lmo3* and/or *Hen2* in neural crest cells, from which neuroblastoma develops, we selected *Wnt1* promoter since it was reported that *Wnt1* is expressed in migrating neural crest cells and the central nervous system [5]. The transgene *HA-Lmo3* or *FLAG-Hen2* was inserted into the expression vector pWEXP-3C, which was kindly supplied by Dr. McMahon (Harvard Univ.) [4]. Transgenic lines were confirmed to express transgenes by RT-PCR using cDNA prepared

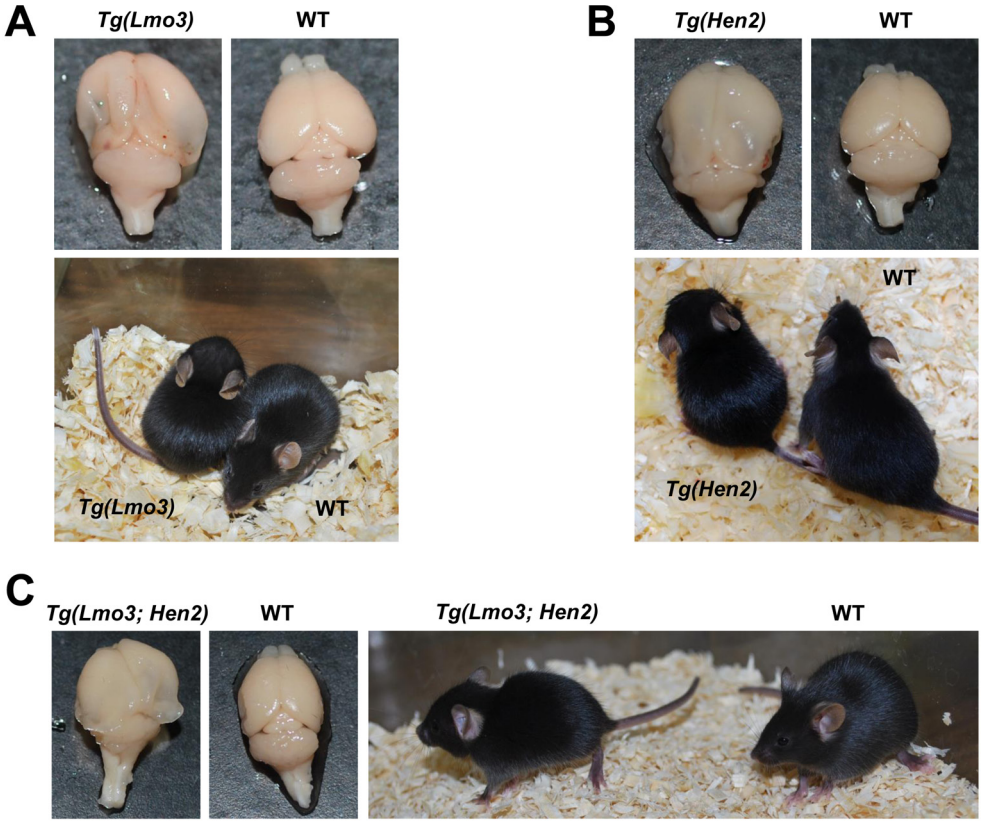

Fig. 2. Hydrocephalus develops in transgenic mice expressing *Lmo3* and/or *Hen2* under the control of *Wnt1* promoter. Brains were prepared from heterozygous *Lmo3* and *Hen2* transgenic mice (*Tg (Lmo3)* and *Tg (Hen2)*) and their littermate wild-type (WT) mice (A and B). Hydrocephalus developed at about 3 weeks of age in *Tg (Lmo3)* and *Tg (Hen2)* (A and B) and heterozygous double-transgenic mice (*Tg (Lmo3; Hen2)*) (C).

from tissues (eyes of P0 mice and E9.5 embryos) that express *Wnt1* as templates (Fig. 1).

Hydrocephalus developed in transgenic mice expressing HA-Lmo3 and/or FLAG-Hen2 under the control of Wnt1 promoter

Hydrocephalus occurs in heterozygous C57BL/6J-*Tg*(*Wnt1-HA-Lmo3*) and C57BL/6J-*Tg*(*Wnt1-FLAG-Hen2*) mice (*Tg (Lmo3)* and *Tg (Hen2)*) and also heterozygous double- transgenic mice (*Tg (Lmo3; Hen2)*) at about 3 weeks of age or earlier (Fig. 2). The frequencies of hydrocephalus in each of the wild type, single- or double-transgenic mice were 0% in wild type, 13% in *Tg (Hen2)*, 9% in *Tg (Lmo3)* and 100% in *Tg (Lmo3; Hen2)* mice (Fig. 3). The frequencies of hydrocephalus in each of the single- or double-transgenic mice were significantly higher as compared with wild type mice by Fisher's test (WT vs. *Tg (Hen2)*, $P=7.567 \times 10^{-11}$; WT vs. *Tg (Lmo3)*, $P=7.351 \times 10^{-8}$) (Fig. 3). According to the Jackson Laboratory, the frequency of hydrocephalus is under 0.01% in mice with the genetic background C57BL/6J. Since the frequencies in *Tg (Lmo3)* and *Tg (Hen2)* mice were higher than that in the wild type mice, each gene may be involved in the development of hydrocephalus. Furthermore, all *Tg (Lmo3; Hen2)* mice developed hydrocephalus. The difference of frequency between *Tg (Lmo3)* and *Tg (Hen2)* was not significant by Fisher's test ($P=0.329$). However the frequency in *Tg (Lmo3; Hen2)* was significantly higher than that in *Tg (Lmo3)* or *Tg (Hen2)* (*Tg (Lmo3; Hen2)* vs. *Tg (Hen2)*, $P=3.252 \times 10^{-9}$; *Tg (Lmo3; Hen2)* vs. *Tg (Lmo3)*, $P=2.948 \times 10^{-11}$; Fisher's test). These results suggest that LMO3 and HEN2 may have synergistic effects on the development of hydrocephalus.

Causes of hydrocephalus are not only aqueduct stenosis but also disorder of cerebral development

Hydrocephalic brains were examined by HE and im-

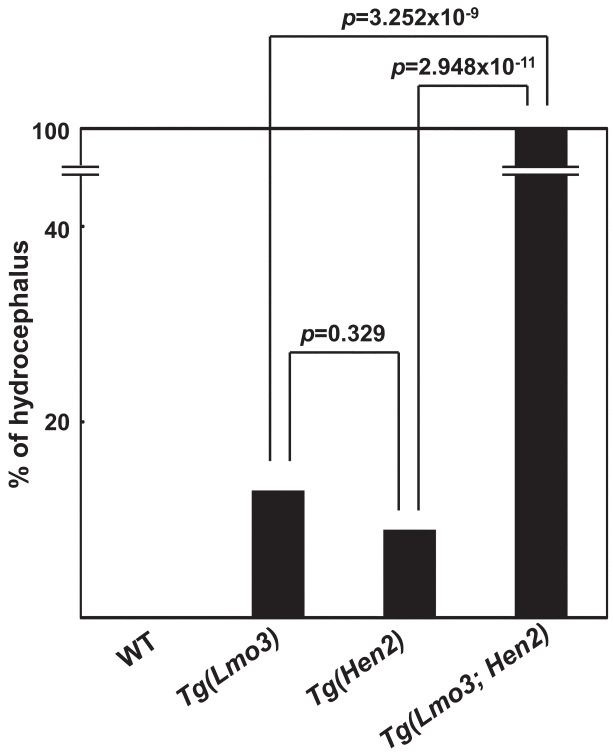

Fig. 3. Frequencies of hydrocephalus in *Tg (Lmo3)* and *Tg (Hen2)* are higher than that in the wild type and all *Tg (Lmo3; Hen2)* mice develop hydrocephalus. Frequencies were calculated by dividing number of hydrocephalic mice (single-, double-transgenic mice or WT mice) by total number of transgenic mice or WT mice. The *P*-value was calculated by Fisher's test.

munohistochemical staining in order to evaluate the pathogenetic mechanisms of hydrocephalus. In hydrocephalic brains of the three genotypes, the cerebral aqueduct was stenotic compared with that in the wild-type (WT) littermates (Figs. 4A, 4B and Table 1). The average area of the cerebral aqueduct in hydrocephalic brain sections was smaller (0.0038 mm^2) than that in WT mice brains (0.0078 mm^2). However, in double-transgenic mouse brain, the extent of stenosis was milder. In order to analyze pathogenesis of aqueduct stenosis, the periaqueductal gray matter was precisely evaluated by immunohistochemistry with antibodies against marker proteins for neurons or glial cells (NEUN: neurons, GFAP: astrocytes, OLIG2: oligodendrocytes or progenitor cells of astrocytes/oligodendrocytes, IBA1: microglia; Fig. 5). There were no differences in cellular components between hydrocephalic and WT mouse brains. The hydrocephalus phenotype was observed in *Tg (Lmo3; Hen2)* mouse embryos at E18.5 (Fig. 4C), but not at E13.5 *Tg (Lmo3; Hen2)* mouse ones (data not shown).

Expression of Hes1 did not change and Expression of Mash1 was slightly decreased in the head tissue of double transgenic mice

Whole head tissue of *Tg (Lmo3; Hen2)* were then examined by RT-PCR at E13.5 in order to check the expression of the transcriptional target of *Lmo3* and *Hen2*, *Mash1* and the transcriptional mediator of *Mash1*, *Hes1*. The transgene *HA-Lmo3* was expressed as well as *FLAG-Hen2* in *Tg (Lmo3; Hen2)* mice. Endogenous *Mash1* and *Hes1* expression was then checked. As a result, no change was detected compared to the wild type control in *Hes1* expression (Fig. S1). On the other hand, expression of *Mash1* was slightly decreased compared to the wild type (Fig. S1). Previously, overexpression of both LMO3 and HEN2 were shown to attenuate HES1 function, resulting in the induction of *MASH1* in neuroblastoma cell lines [8]. LMO3 and HEN2 attenuate HES1 function, but *HES1* expression does not decrease necessarily [8]. *Mash1* expression in embryonic brains of *Tg (Lmo3; Hen2)* was different from the previous *in vitro* study. This is possibly because the negative feedback interferes with *Mash1* expression [8] or other transcriptional targets of *Lmo3* and *Hen2* could exist *in vivo*. Comprehensive study, such as Chip-seq analysis with LMO3 and HEN2 antibodies would give us clear answers to this question in the future study.

Discussion

In this study, transgenic mice of *Lmo3* and *Hen2* were established in order to study their roles in the development and tumorigenesis of the nervous system using promoter of *Wnt1*, which is expressed in neural crest cells and the central nervous system.

The frequencies of hydrocephalus in *Tg (Lmo3)* and *Tg (Hen2)* mice were higher than in the wild type ones, respectively (Fig. 3). Furthermore, all *Tg (Lmo3; Hen2)* mice developed hydrocephalus. Therefore, it seems that each gene is involved in the development of hydrocephalus and they have a synergistic effect on this. In *Tg (Lmo3; Hen2)* mouse brain, the extent of stenosis was mild (Table 1 and Fig. 4B). The cellular component surrounding the periaqueductal gray matter showed no significant difference between hydrocephalus and non-hydrocephalus from any genotypes, suggesting that reac-

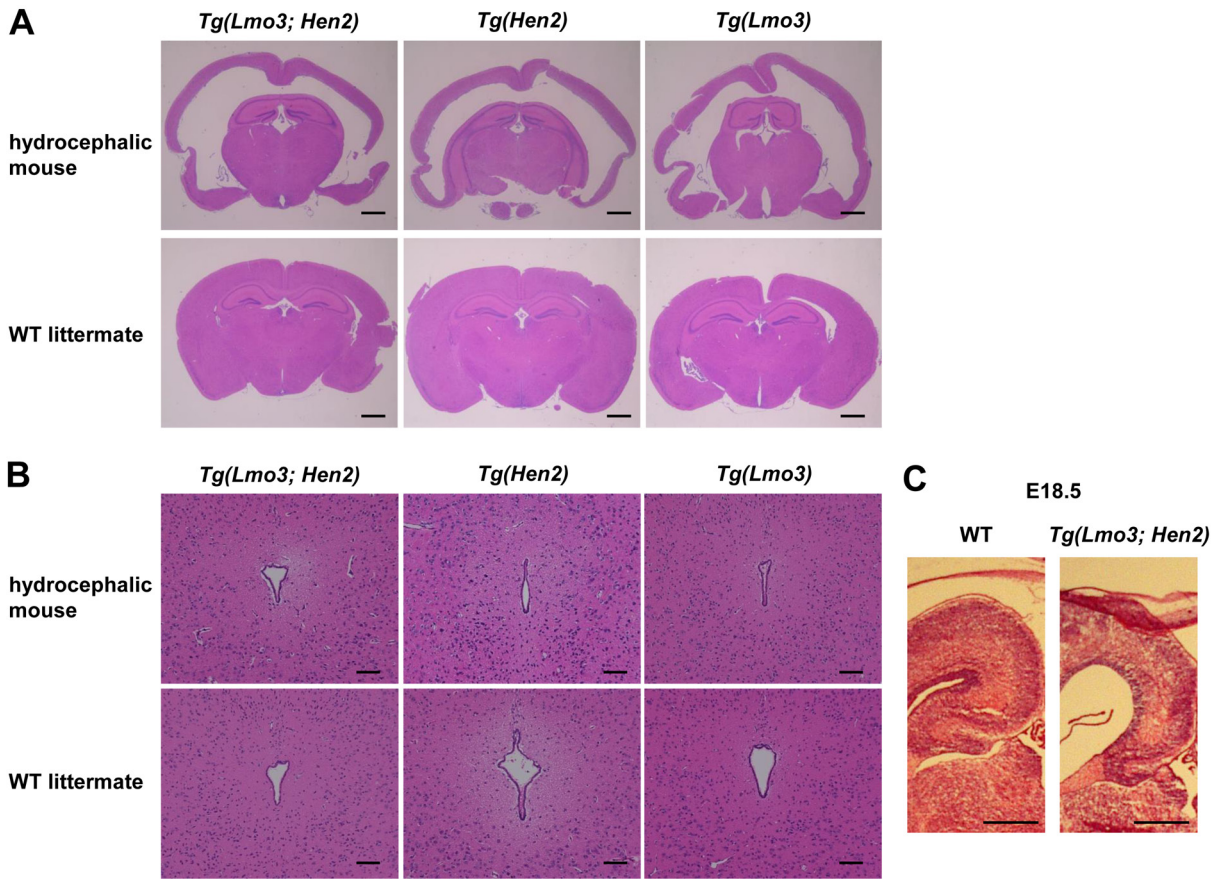

Fig. 4. Causes of hydrocephalus are not only aqueduct stenosis but also disorder of cerebral development. Hydrocephalic brains were examined by HE staining. In hydrocephalous brains of *Tg (Lmo3)* and *Tg (Hen2)* mice (A and B), cerebral aqueduct was stenotic compared with that of the wild-type littermates (B). In *Tg (Lmo3; Hen2)* mice with hydrocephalous brain (A and B), the extent of stenosis was mild (B). Hydrocephalus developed in *Tg (Lmo3; Hen2)* mice at E18.5 (C). Scale bars=1,500 μm (A),=300 μm (B) and=600 μm (C), respectively.

Table 1. Summary of phenotypes of brains in hydrocehalic mice and their wild-type littermates

Genotype	HE stain		Density of immunoreactive cells (/mm^2)			
	general	aqueductus cerebri	NeuN	GFAP	Olig2	Iba1
Tg (Lmo3; Hen2)	severe hydrocephalus	mildly stenotic	24	56	200	100
WT littermate	normal	normal	16	58	373	108
Tg (Hen2)	severe hydrocephalus	stenotic	30	98	247	80
WT littermate	normal	normal	30	134	163	72
Tg (Lmo3)	severe hydrocephalus	stenotic	24	72	263	90
WT littermate	mild hydrocephalus	normal	32	68	280	100

Average areas of aqueductus cerebri from 4 brains were 0.0038 mm^2 and 0.0078 mm^2 in hydrocephalic mice and their wild-type littermates, respectively. Numerical density of immunoreactive cells was counted (/mm^2).

tive changes might not be caused by inflammation, circulation disorder, etc. in the periaqueductal gray.

Hydrocephalus occurred in E18.5 *Tg (Lmo3; Hen2)* embryos (Fig. 4C), but not in E13.5 *Tg (Lmo3; Hen2)* ones (data not shown). These results suggest that the causes of the hydrocephalus are not only aqueduct stenosis but also abnormalities during cerebral development. From E12.5 to E16.5, proliferation and migration of neural progenitors proceed in murine fetal cortices. Thus, *Lmo3* and *Hen2* might interfere with development

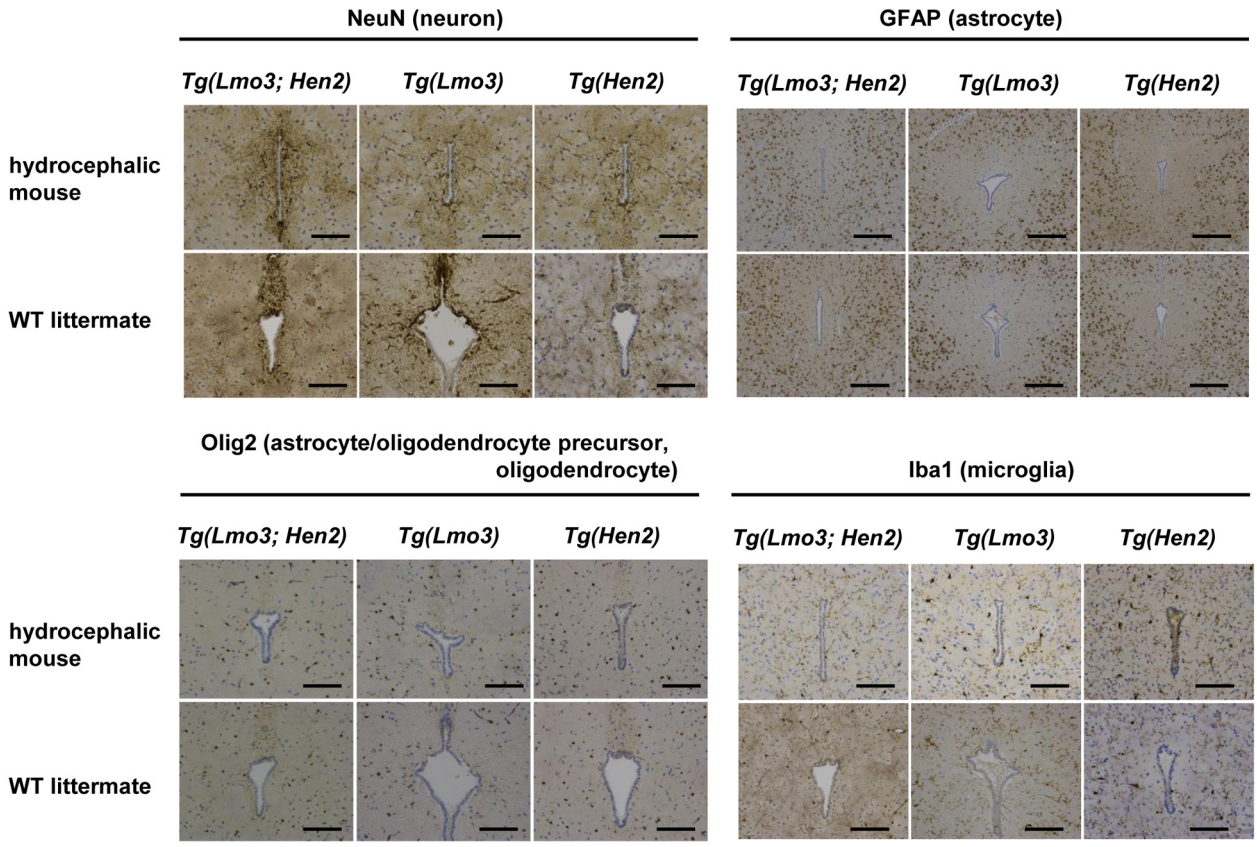

Fig. 5. Immunohistochemical analyses showed no significant differences between phenotypesDistribution and number of neurons (NEUN), astrocytes (GFAP), oligodendrocytes or progenitor cells of astrocytes/oligodendrocytes (OLIG2) and microglia (IBA1) around the aqueduct did not differ between hydrocephalic mice and their wild-type littermates. Scale bar=300 μm.

of the neocortex and consequently the formation of the neocortex may be disordered. In order to clarify the causes of this, analysis of fetal brain development is required.

In *Robo1/2* knockout mice (*Robo1/2*$^{-/-}$), the thickness of cortex and density of ventricular zone (VZ) were decreased [3]. Loss of *Robo1/2* function leads to a depletion of VZ progenitors and to an abnormal increase in the numbers of intermediate progenitor cells (IPCs) in the developing cerebral cortex. The expression of *Hes1* was significantly reduced in the cortex of E12.5 *Robo1/2*$^{-/-}$ compared with that in controls. A reduction in *Hes1* levels could explain the decreased level of VZ mitosis and the increase in IPCs found in the *Robo1/2*$^{-/-}$ cortex because *Hes1* expression is thought to maintain the status of progenitor cells in the VZ. We have already reported that there could be a functional relationship between LMO3/HEN2 and MASH1 in neuroblastoma, and found that LMO3/HEN2 attenuates HES1 function and enhances the transactivation of *MASH1*, leading to an aggressive phenotype of neuroblastoma [8]. Therefore, in double-transgenic mice, they cooperate and inhibit HES1 function and could induce aberrant neurogenesis, decrease cortical thickness and induce hydrocephalus. To clarify this relationship between hydrocephalus and the inhibitory effects of *Lmo3* and *Hen2* on HES1, analysis of cortical development is necessary.

It was suggested that oncogenic *Lmo3* could cooperate with *Hen2* to induce aberrant neurogenesis, hydrocephalus, in mice. This may occur by a similar mechanism to that shown in neuroblastoma, disorder of peripheral nervous system development. The expression levels of *Lmo3* and/or *Hen2* could determine the fate of stem cells by inhibiting HES1 function during nervous system development and might be a trigger of aberrant neurogenesis *in vivo*. This might be a common mechanism that induces aberrant neuronal development, leading to neuronal disorder in central and peripheral nervous systems.

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