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Hes1 and *Hes5* are required for differentiation of pituicytes and formation of the neurohypophysis in pituitary development

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ABSTRACT

The pituitary gland is a critical endocrine organ regulating diverse physiological functions, including homeostasis, metabolism, reproduction, and growth. It is composed of two distinct entities: the adenohypophysis, including the anterior and intermediate lobes, and the neurohypophysis known as the posterior lobe. The neurohypophysis is composed of pituicytes (glial cells) and axons projected from hypothalamic neurons. The adenohypophysis derives from Rathke's pouch, whereas the neurohypophysis derives from the infundibulum, an evagination of the ventral diencephalon. Molecular mechanisms of adenohypophysis development are much better understood, but little is known about mechanisms that regulate neurohypophysis development. *Hes* genes, known as Notch effectors, play a crucial role in specifying cellular fates during the development of various tissues and organs. Here, we report that the ventral diencephalon fails to evaginate resulting in complete loss of the posterior pituitary lobe in *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos. In these mutant mice, progenitor cells are differentiated into neurons at the expense of pituicytes in the ventral diencephalon. In the developing neurohypophysis, the proliferative zone is located at the base of the infundibulum. Thus, *Hes1* and *Hes5* modulate not only maintenance of progenitor cells but also pituicyte *versus* neuron fate specification during neurohypophysis development.

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1. Introduction

The pituitary gland is a critical endocrine organ, which is essential for homeostasis, metabolism, reproduction, and growth (Davis et al., 2013; Hojo et al., 2008; Kita et al., 2007; Zhu et al., 2005; Zhu and Rosenfeld, 2004). It is composed of two distinct entities: the adenohypophysis and the neurohypophysis. The adenohypophysis includes the anterior and intermediate lobes, and the neurohypophysis constitutes the posterior lobe. The anterior lobe contains five hormone-producing cell types: corticotropes secreting adrenocorticotrophic hormone (ACTH), thyrotropes secreting thyroid-stimulating hormone (TSH), gonadotropes secreting luteinizing hormone (LH) and follicle-stimulating hormone (FSH), somatotropes secreting growth hormone (GH) and lactotropes secreting prolactin (PRL), and the intermediate lobe contains one hormone-producing cell type: melanotropes secreting alpha melanocyte-stimulating hormone (α-MSH). By contrast, the neurohypophysis is composed of pituicytes and axons projected from hypothalamic neurons that release arginine vasopressin and oxytocin. Unlike other pituitary hormonal cells, pituicytes are defined as glial cells.

During embryonic development, the pituitary gland originates from two separate germinal tissues. The adenohypophysis derives from Rathke's pouch, whereas the neurohypophysis derives from the infundibulum, an evagination of the ventral diencephalon (Hojo et al., 2008; Kaufmann, 1992; Kita et al., 2007). The oral ectoderm thickens and invaginates to form Rathke's pouch at embryonic day 8.5 (E8.5) in mice. The dorsal portion of Rathke's pouch directly contacts the ventral diencephalon, which evaginates at E10 to form the infundibulum. Rathke's pouch separates from the oral ectoderm and further develops and differentiates. It had been believed that the stratified appearance of endocrine cells in the anterior lobe results from an ordered specification of cell types by interacting gradients of bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) signaling. Recently, Davis et al. (2010) have proposed a new model for pituitary cell specification that focuses on intrinsic factors including the BMP, WNT, and Notch signaling pathways. These factors are necessary for cell specification between E11.5 and E13.5, and cell–cell communication likely plays an important role in regulating this process (Davis et al., 2010, 2013).

Molecular mechanisms of adenohypophysis development are much better understood, but little is known about mechanisms that regulate neurohypophysis development (Davis et al., 2013; Hojo et al., 2008; Zhu et al., 2005; Zhu and Rosenfeld, 2004). Previous studies have reported that deletion of the homeobox gene *Nkx2.1* leads to loss of the infundibulum during pituitary development (Kimura et al., 1996; Takuma et al., 1998). Deletion of the homeobox gene *Lhx2* also results in loss of the infundibulum (Zhao et al., 2010). In *SOX3*-deficient embryos, the evagination of the infundibulum is less pronounced (Rizzoti et al., 2004). However, mechanisms of differentiation of pituicytes have not been examined at all in these studies.

In many organs, cell proliferation and differentiation are antagonistically regulated by multiple basic helix–loop–helix (bHLH) genes (Kageyama et al., 2005; Ohsawa and Kageyama, 2008). The repressor-type bHLH genes include *Hes* genes, homologs of *Drosophila hairy and Enhancer of split* [*E(spl)*]. Notch is a transmembrane protein and activated by its ligands such as Delta. Although Notch signaling is not the sole regulator of Hes factors, Hes1 and Hes5 are essential effectors for Notch signaling. *Hes* genes regulate the maintenance of stem cells and progenitors, and control the normal timing of cell differentiation. In addition, *Hes* genes regulate binary cell fate decision in many tissues and organs: *Hes* genes promote astrocyte *versus* neuron, enterocyte *versus* non-enterocyte, billiary cell *versus* hepatocytic cell, exocrine cell *versus* endocrine cell, and T cell *versus* B cell fate decisions (Kageyama et al., 2007, 2008a, 2008b, 2009).

The analysis of targeted mouse mutants has demonstrated roles of the Notch signaling pathway in adenohypophysis development (Davis et al., 2010; Himes and Raetzman, 2009; Hojo et al., 2008; Monahan et al., 2009; Nantie et al., 2014; Raetzman et al., 2004, 2007; Zhu and Rosenfeld, 2004; Zhu et al., 2007). We have reported that in conditional *Hes1*; *Hes5* double-mutant mice, the pituitary gland is severely hypoplastic and dysmorphic (Kita et al., 2007). In the absence of *Hes1* and *Hes5*, cell differentiation is accelerated and progenitors are prematurely differentiated in the developing pituitary gland. Although we have also reported that the neurohypophysis is lost in conditional *Hes1*; *Hes5* double-mutant mice, the molecular mechanism of this phenotype was not investigated at all. *Hes1* also regulates migration of hypothalamic neurons and axonal projection to the pituitary gland (Aujla et al., 2011). Here, we report that *Hes1* and *Hes5* modulate the differentiation of pituicytes, and are essential for formation of the neurohypophysis during mouse pituitary development.

2. Results

2.1. *Hes1 is compensated by Hes5 in the developing ventral diencephalon*

We have previously reported the expression pattern of *Hes1* in mice during pituitary development (Kita et al., 2007). Here, we examined the expression pattern of *Hes1* in the developing ventral diencephalon and neurohypophysis. At E10.5, *Hes1* is strongly expressed in the ventral diencephalon (Kita et al., 2007). At E12.5, *Hes1* expression was diminished in the evagination of the ventral diencephalon (the infundibulum) (Fig. 1A). In this region, *Hes1* expression was restricted in the periluminal side of the base of the infundibulum (Fig. 1A, arrowhead). At E14.5, *Hes1* expression is further downregulated in the ventral diencephalon (Kita et al., 2007). These findings suggest that *Hes1* may control the evagination of the ventral diencephalon and the development of the neurohypophysis.

Since we have reported that *Hes5* compensates *Hes1* during the development of various organs (Hatakeyama et al., 2004; Kita et al., 2007; Kitagawa et al., 2013), we next examined *Hes5* expression in the developing ventral diencephalon. In wild-type mice, *Hes5* was not detected in the ventral diencephalon (Fig. 1B). However, *Hes5* was upregulated in the ventral diencephalon of *Hes1*-null mice (Fig. 1C),

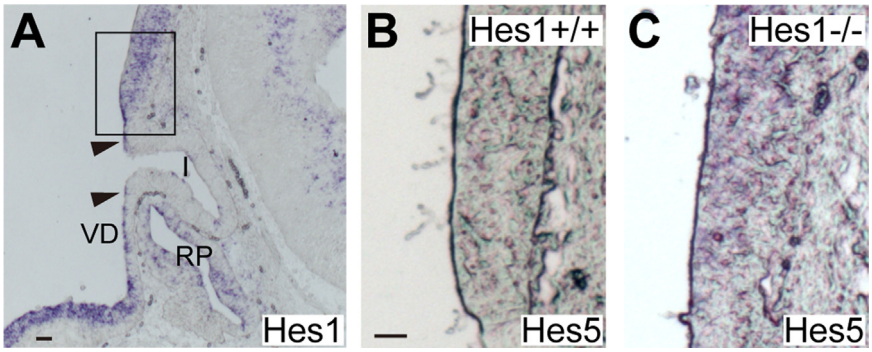


Fig. 1 – Expression of *Hes1* and *Hes5* in the developing pituitary posterior gland. (A) *In situ* hybridization of *Hes1*. At E12.5, *Hes1* was strongly expressed in the ventral diencephalon. However, *Hes1* expression was faint in the infundibulum. At the base of the infundibulum, *Hes1* expression was restricted in the periluminal side of the ventral diencephalon (arrowheads). The boxed area in (A) shows the approximate regions examined in (B) and (C). (B and C) *In situ* hybridization of *Hes5*. *Hes5* was not detected in the ventral diencephalon of the control (B). However, in *Hes1*-null mice, *Hes5* was upregulated in the ventral diencephalon (C). VD, ventral diencephalon; RP, Rathke's pouch; I, infundibulum. Bars, 100 μm (A, B and C).

suggesting that *Hes5* can compensate *Hes1* for the development of the neurohypophysis. Therefore, to understand the roles of *Hes* genes in the development of the neurohypophysis, we decided to analyze *Hes1*; *Hes5* double-mutant mice. However, we cannot examine pituitary development of *Hes1*; *Hes5* double mutant mice, because these mutant embryos die by E11 (Ohtsuka et al., 1999). Since it has been reported that Notch signaling molecules such as Notch1 and Dll4 have haploinsufficiency, we expected that *Hes5* would compensate *Hes1* in a dosage-sensitive manner (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos can be alive just before birth, whereas *Hes1*$^{-/-}$; *Hes5*$^{-/-}$ mutant embryos die by E11. Thus, we decided to analyze *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos to understand roles of *Hes1* and *Hes5* in neurohypophysis development.

2.2. *The ventral diencephalon fails to evaginate in Hes1*$^{-/-}$*; Hes5*$^{+/-}$ *mutant embryos*

We first examined the development of the posterior pituitary lobe at E12.5. In *Hes1*-null embryos, the evagination of the ventral diencephalon was morphologically normal but small compared with the control (Fig. 2A and B). However, in *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the evagination was completely lost (Fig. 2C). To investigate mechanisms of the failure of evagination, we analyzed cell proliferation by Ki67 immunostaining (Fig. 2D–G). In the infundibulum of the control, Ki67^{+} cells were significantly decreased compared to the adjacent ventral diencephalon (Fig. 2D and G). At the tip of the infundibulum, no Ki67^{+} cell was detected. In the infundibulum, bromodeoxyuridine (BrdU) incorporated cells were also reduced (details will be described later). These findings suggest that cells have stopped proliferating in the infundibulum, especially in its distal portion, during neurohypophysis development. In *Hes1*-null embryos, Ki67^{+} cells were significantly decreased in the infundibulum compared with the control (Fig. 2D, E and G), suggesting that cell proliferation is decreased in the developing infundibulum. BrdU incorporated cells were also reduced in *Hes1*-null embryos (details will be described later). In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the evagination was not observed, and Ki67^{+} cells were detected in the ventricular layer of the ventral diencephalon (Fig. 2F and G). Thus, *Hes1* and *Hes5* are essential for the maintenance of progenitor cells in the ventral diencephalon during neurohypophysis development.

2.3. *Progenitor cells differentiate into neurons at the expense of pituicytes in Hes1*$^{-/-}$*; Hes5*$^{+/-}$ *mutant embryos*

To investigate mechanisms of the failure of evagination in *Hes* mutants, we performed immunostaining with anti-S100β and anti-Tuj1 antibodies at E12.5. S100β is a good immunohistochemical marker for both pituicytes and astrocytes (Wei et al., 2009), and Tuj1 is a marker for differentiating neurons. In control mice, S100β^{+} cells were observed in the infundibulum and in the outer layer of the ventral diencephalon (Fig. 3A). In the infundibulum, S100β^{+} cells were notably detected in the distal region. These S100β^{+} cells in the infundibulum may be pituicytes. S100β^{+} cells were also observed in the ventral diencephalon. Although there is no definitive evidence, these S100β^{+} cells in the ventral diencephalon may be astrocytes. In *Hes1*-null embryos, S100β^{+} cells were decreased compared with the control (Fig. 3A and B). In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, S100β^{+} cells were not detected (Fig. 3C). These results show that progenitor cells do not differentiate into pituicytes or astrocytes in *Hes* mutant mice. In control mice of E12.5, Tuj1^{+} cells (neurons) were slightly observed in the ventral diencephalon and the infundibulum (Fig. 3D). In *Hes1*-null embryos, Tuj1^{+} cells were increased in the ventral diencephalon (Fig. 3E). Interestingly, in addition to the ventral diencephalon, Tuj1^{+} cells were increased in the infundibulum in *Hes1*-null embryos (Fig. 3E). In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, Tuj1^{+} cells were further increased and the ventral diencephalon was thickened (Fig. 3F). These findings show that progenitor cells differentiate into neurons in *Hes* mutant mice. Taken together, in the absence of *Hes* genes, progenitor cells may differentiate into neurons at the expense of pituicytes or astrocytes.

We next performed immunostaining with an anti-calbindin antibody. Calbindin is an immunohistochemical marker for both neurons and immature pituicytes (Abe et al., 1991; Miyata et al., 2000), whereas S100β is a marker for mature

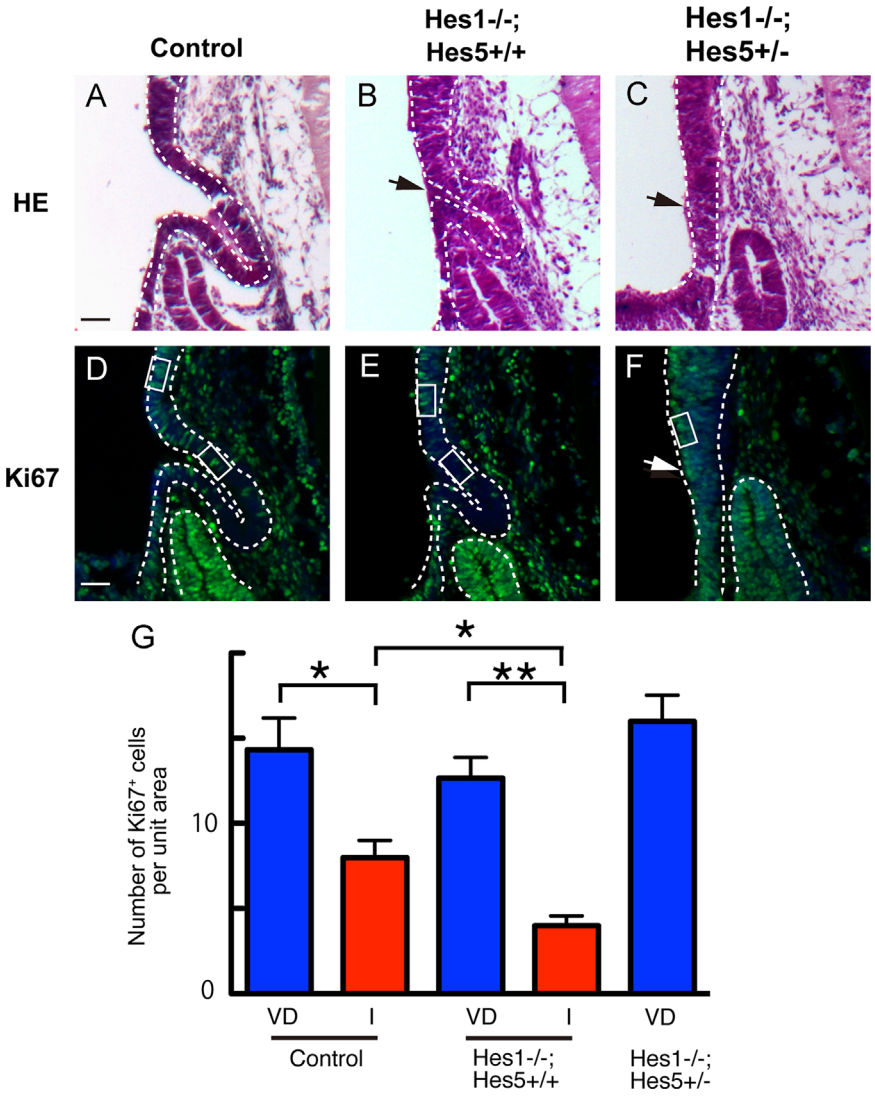


Fig. 2 – The ventral diencephalon fails to evaginate in *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos. (A–C) Midsagittal sections of E12.5 embryos were stained with HE. In *Hes1*-null embryos, the evagination of the ventral diencephalon was morphologically normal but small compared with the control (A and B). In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the evagination was completely lost (C, arrow). (D) In the infundibulum of the control, Ki67^{+} cells were decreased, and in the tip of the infundibulum, no Ki67^{+} cell was detected. (E) In *Hes1*-null embryos, Ki67^{+} cells were further decreased in the infundibulum compared with the control. (F) In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the evagination was not observed, and Ki67^{+} cells were detected in the ventricular layer of the ventral diencephalon. (G) Quantification of Ki67^{+} cells. Ki67^{+} cells were counted in the unit area (boxed areas in (D–F)). Three independent embryos of each genotype were examined. Ki67^{+} cells were significantly decreased in the infundibulum compared to the adjacent ventral diencephalon in both control and *Hes1*-null mice ($^{*}p<0.05$ and $^{**}p<0.005$, *t*-test). Moreover, Ki67^{+} cells were significantly decreased in the infundibulum of *Hes1*-null embryos compared with the control ($^{*}p<0.05$, *t*-test). Bars, 50 μm (A–C and D–F).

pituicytes. Immature pituicytes are weakly and transiently positive for calbindin, but neurons are strongly positive for it (Abe et al., 1991). In control mice of E12.5, the proximal region of the infundibulum was weakly positive for calbindin (Fig. 4A), suggesting that these calbindin^{+} (calbindin^{low}) cells may be immature pituicytes. A few cells in the outer layer of the ventral diencephalon were strongly positive for calbindin (Fig. 4A, arrowheads), suggesting that these calbindin^{+} (calbindin^{high}) cells may be neurons. Mature (S100β^{+}) and immature (calbindin^{low}) pituicytes were observed in the distal and proximal infundibulum, respectively (Figs. 3A and 4A). In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the evagination was not observed, and the outer layer of the ventral diencephalon was strongly positive for calbindin (calbindin^{high}) (Fig. 4B, arrowheads). These results are consistent with findings on Tuj1 staining (Fig. 3D and F), suggesting that these calbindin^{high} cells may be neurons. These results indicate that, in the absence of *Hes* genes, progenitor cells differentiate into neurons at the expense of pituicytes.

To further characterize the developing infundibulum, we performed immunostaining with an anti-*SOX2* antibody. *SOX2* is a marker for progenitor cells (Fauquier et al., 2008;

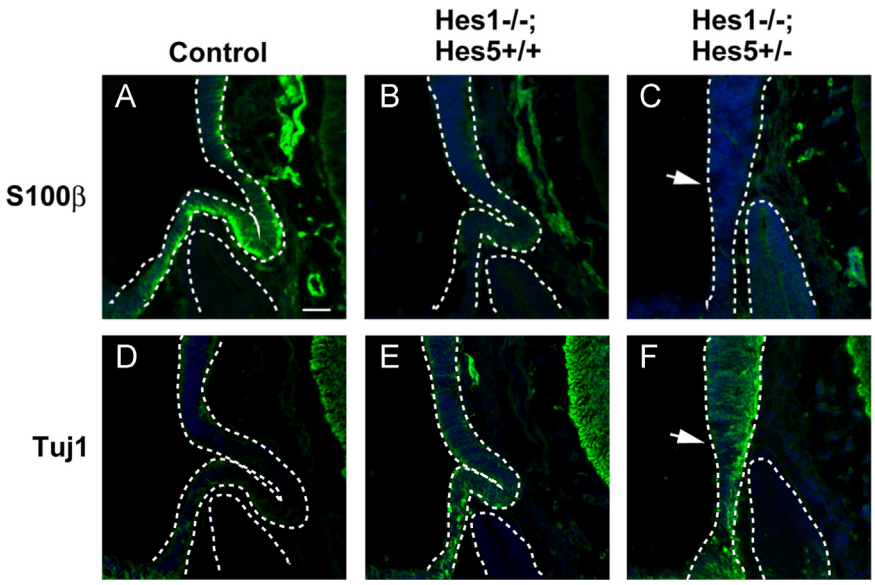


Fig. 3 – Progenitor cells differentiate into neurons at the expense of pituicytes in *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos. (A–C) Immunostaining with an anti-S100β antibody. (A) In control mice, S100β^{+} cells were observed in the infundibulum and in the outer layer of the ventral diencephalon. In the infundibulum, S100β^{+} cells were notably detected in the distal region. (B) In *Hes1*-null embryos, S100β^{+} cells were decreased compared with the control. (C) In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the evagination was lost, and S100β^{+} cells were not detected. (D–F) Immunostaining with an anti-Tuj1 antibody. (D) In control mice of E12.5, Tuj1^{+} cells were slightly observed in the ventral diencephalon and the infundibulum. (E) In *Hes1*-null embryos, Tuj1^{+} cells were increased in the ventral diencephalon. In addition to the ventral diencephalon, Tuj1^{+} cells were increased in the infundibulum in *Hes1*-null embryos. (F) In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, Tuj1^{+} cells were further increased and the ventral diencephalon was thickened. Bars, 50 μm (A–F).

Langer et al., 2012; Trowe et al., 2013). In control mice of E12.5, *SOX2*$^{+}$ cells were observed in both the ventral diencephalon and the infundibulum (Fig. 4C). It is likely that not only progenitor cells and but also immature pituicytes are positive for *SOX2*. In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the evagination was not observed (arrow), and the outer layer of the ventral diencephalon was negative for *SOX2* (Fig. 4D). This *SOX2*-negative region is positive for Tuj1 and strongly positive for calbindin (Figs. 3F and 4B), suggesting that progenitor cells are differentiated into neurons at the expense of pituicytes in the absence of *Hes* genes.

To investigate whether cell death is involved in defects in the infundibulum, we performed terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. Both control and *Hes*-mutant embryos were negative for TUNEL assay in the infundibulum and the ventral diencephalon (Fig. 4E and F), suggesting that apoptosis is not the cause of hypoplasia or lack of the infundibulum in *Hes* mutant mice.

Taken together, these results show that, in the absence of *Hes* genes, progenitors may differentiate into neurons at the expense of pituicytes, resulting in loss of the infundibulum. Pituicytes are more differentiated in the distal than in the proximal part of the infundibulum. It is likely that the ventral diencephalon cannot evaginate and may be thickened in *Hes* mutant mice, because neurons cannot induce an evagination like pituicytes.

2.4. *Progenitor cells differentiate into pituicytes at the base of the infundibulum*

To further investigate mechanisms of formation of the infundibulum, we examined the proliferation patterns of differentiating cells using pulse chase labeling of growing DNA chains with the nucleoside analogs idiodeoxyuridine (IdU) and BrdU (Ward et al., 2005). Although IdU and BrdU are similar thymidine analogs, we can distinguish them by immunohistochemisty. We can investigate the cell proliferation patterns by sequential injections of IdU and BrdU at different times of development. We labeled cells at E11.5 by IdU and at E12.5 by BrdU, and collected embryos one hour after the last injection. The anti-IdU antibody detects both IdU and BrdU, but the anti-BrdU antibody detects only BrdU. Therefore, cells that were labeled at E11.5 with IdU and at E12.5 with BrdU appear *green* when visualized with the anti-IdU antibody and Alexa 488 fluorescence (Fig. 5A–C). Cells that were labeled at E12.5 with BrdU appear *red* when visualized with the anti-BrdU antibody and Alexa 594 fluorescence (Fig. 5D–F). When these images are overlaid, the BrdU-labeled cells appear *yellow* as a result of the combination of the *red* and *green* fluorescence, and the IdU-labeled cells appear *green* (Fig. 5G–L).

In control mice of E12.5, BrdU-incorporated cells (*yellow*) were located mainly in the ventral diencephalon, but scarcely in the infundibulum (Fig. 5A, D, G and J), suggesting that cells in the infundibulum have stopped proliferating at E12.5. Cells

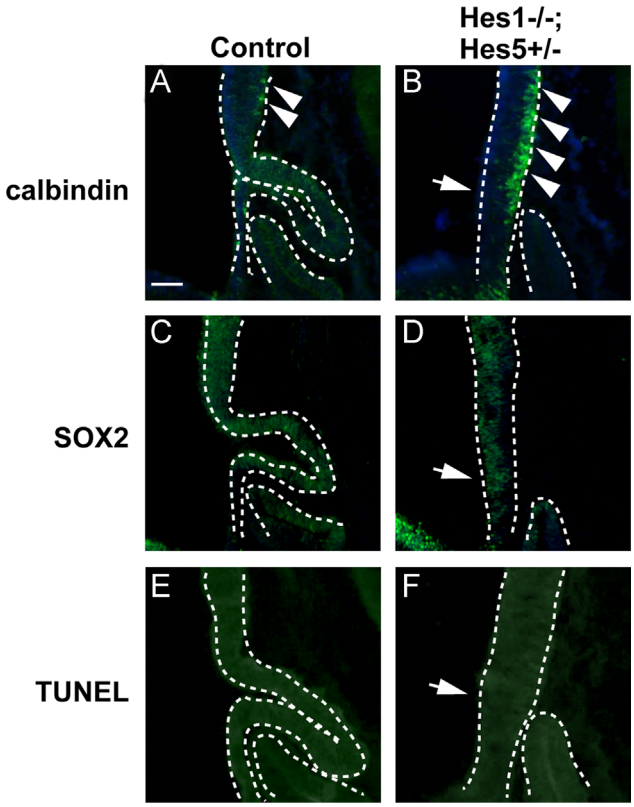


Fig. 4 – Progenitor cells differentiate into neurons at the expense of pituicytes in *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos. (A–D) Midsagittal sections of E12.5 embryos were stained with anti-calbindin and anti-*SOX2* antibodies. Immature pituicytes are weakly and transiently positive for calbindin, whereas neurons are strongly positive for it. (A) In control mice, the proximal region of the infundibulum was diffusely and weakly positive for calbindin, suggesting that these calbindin^{+} (calbindin^{low}) cells may be immature pituicytes. A few cells in the outer layer of the ventral diencephalon were strongly positive for calbindin (arrowheads), suggesting that these calbindin^{+} (calbindin^{high}) cells may be neurons. (B) In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the evagination was not observed (arrow), and the outer layer of the ventral diencephalon were strongly positive for calbindin (calbindin^{high}). (C) In control mice, *SOX2*$^{+}$ cells were observed in both the ventral diencephalon and the infundibulum. (D) In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the evagination was not observed (arrow), and the outer layer of the ventral diencephalon was negative for *SOX2*. (E and F) TUNEL assay. Both control and *Hes*-mutant embryos were negative for TUNEL assay in the infundibulum and the ventral diencephalon. Bars, 50 μm (A–F).

that incorporated IdU at E11.5 but did not incorporated BrdU at E12.5 (*green*) were located mainly in the proximal portion of the infundibulum (Fig. 5A, D, G and J). These cells (*green*) were significantly decreased in the tip of the infundibulum compared to the proximal portion (Fig. 5M). These findings show that the proliferative zone is located in the proximal portion of the infundibulum, but not in its apex.

In *Hes1*-mutant embryos, the evagination of the ventral diencephalon was observed but small compared with the control. Cells that incorporated IdU at E11.5 (*green*) were located in the proximal portion of the infundibulum but were significantly reduced compared with the control (Fig. 5B, E, H, K and M). In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the evagination was completely lost. IdU-incorporated cells were located in the outer layer of the infundibulum (Fig. 5C, F, I and L). The outer layer of the ventral diencephalon is positive for Tuj1 and strongly positive for calbindin (Figs. 3F and 4B), suggesting that these IdU^{+} Tuj1^{+} calbindin^{high} cells are neurons, and that progenitor cells are differentiated into neurons at the expense of pituicytes in the absence of *Hes* genes. It is likely that neurons cannot induce the evagination like pituicytes.

Taken together, these results show that progenitor cells may differentiate into pituicytes in the proximal portion of the infundibulum, but not in its apex.

2.5. *The neurohypophysis is lost in Hes1*$^{-/-}$*; Hes5*$^{+/-}$ *mutant embryos at E16.5*

Next, we examined the development of the neurohypophysis at E16.5. In *Hes1*-mutant embryos, the posterior pituitary lobe was reduced in size but morphologically normal compared with the control (Fig. 6A and B). In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the posterior pituitary lobe was completely lost (Fig. 6C). In about half of *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, this phenotype was prominent and both the posterior lobe and the pituitary stalk did not form. But in about half of mutants, this phenotype was a little weak and the posterior lobe was lost but the pituitary stalk was truncated and observed (data not shown).

To investigate mechanisms of the development of the posterior pituitary lobe, we performed immunostaining with an anti-S100β antibody. In both *Hes1*-null and control embryos, S100β^{+} cells (pituicytes) were observed in the posterior lobe (Fig. 6D and E). In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the posterior lobe was not detected (Fig. 6F, arrowhead). Next, to investigate the axon that is projected from the hypothalamus to the posterior lobe, we stained with an anti-Tuj1 antibody. In both *Hes1*-null and control embryos, Tuj1 was positive continuously from the ventral diencephalon to the posterior lobe (Fig. 6G and H). The staining pattern of *Hes1*-mull mice was similar to that of the control. In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the posterior lobe did not form (Fig. 6I, arrowhead).

To further characterize the developing neurohypophysis, we performed immunostaining with an anti-cellular retinoic acid binding protein (CRABP) 2 antibody. CRABP2 is a marker for mature pituicytes (Ruberte et al., 1992). In both *Hes1*-null and control embryos, CRABP2^{+} cells (pituicytes) were observed in the posterior lobe (Fig. 6J and K). The staining pattern of CRABP2 was similar to that of S100β. In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the posterior pituitary lobe did not form, and CRABP2^{+} cells were not detected (Fig. 6L, arrowhead). Next, to investigate the projection of the axon from the hypothalamic neuron to the posterior lobe, we stained with an anti-vasopressin antibody. Neurons located in the paraventricular and supraorbital nucleus release vasopressin from the axonal terminals within the neurohypophysis. In both *Hes1*-null and control embryos, vasopressin was positive continuously from the ventral diencephalon to the posterior

lobe (Fig. 6M and N). In *Hes1*-null embryos, the amount of vasopressin was reduced compared with the control. In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the posterior pituitary lobe and the stalk were lost, and vasopressin was detected only in the ventral diencephalon (Fig. 6O).

Taken together, these findings show that the posterior lobe does not form and the pituitary stalk is lost or truncated in the absence of *Hes* genes, and that *Hes* genes are essential for formation of the posterior pituitary lobe.

3. Discussion

3.1. *Progenitors of pituicytes are regulated by the gliogenic activity of Hes genes*

We found that, in *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, progenitor cells differentiate into neurons at the expense of pituicytes, and the ventral diencephalon fails to evaginate resulting in complete loss of the posterior pituitary lobe. In the developing nervous system, *Hes* genes are essential for the maintenance of neural stem cells. In the absence of *Hes* genes, neural stem cells are prematurely differentiated into neurons only and become depleted without generating other cell types (Kageyama et al., 2007, 2008a). In addition to maintaining stem cells, *Hes* genes regulate binary cell fate decisions. For example, *Hes* genes control astrocyte *versus* neuron fate specification: they promote astrocyte formation in the developing nervous system. *Hes* genes promote gliogenesis and inhibit generation of neurons. Therefore, it is likely that *Hes1* and *Hes5* control pituicyte *versus* neuron fate specification in neurohypophysis development, because pituicytes are specialized astroglial cells. The gliogenic activity of *Hes* genes may promote differentiation of pituicytes and formation of the neurohypophysis.

In the neural development, *Hes* genes control the timing of differentiation of neural stem cells by repressing activator-type bHLH genes, such as *Mash1*, *Math* and *Neurogenin* (Kageyama et al., 2008b). It is possible that these transcription factors may act downstream of *Hes* genes during neurohypophysis development, although we have no definitive evidence.

3.2. *The proliferative zone is located at the base of the infundibulum*

The results of our study suggest a possible mechanism of neurohypophysis development (Fig. 7). At E12.5, most cells in the infundibulum are Ki67^{-} and do not incorporate BrdU, indicating that they have already stopped proliferating. In contrast, cells at the base of the infundibulum are Ki67^{+} and incorporate BrdU, suggesting that they may be progenitor cells of pituicytes (red dots in Fig. 7). These progenitor cells of pituicytes are Hes1^{+}, Ki67^{+}, BrdU^{+}, calbindin^{-}, and S100β^{-} (red dots in Fig. 7). The proliferative zone is located at the base of the infundibulum, but not in its apex. Progenitor cells differentiate into immature pituicytes (Hes1^{-}, Ki67^{-}, BrdU^{-}, calbindin^{low}, and S100β^{-}, blue squares in Fig. 6), and then they further differentiate into mature pituicytes (Hes1^{-}, Ki67^{-}, BrdU^{-}, calbindin^{-}, and S100β^{+}, green squares in Fig. 7).

It has been reported that the chick infundibulum derives from two subsets of anterior ventral midline cells. One set remains at the ventral midline and forms the posterior–ventral infundibulum. A second population gives rise to a collar of cells that are capable of extensive proliferation, and forms the anterior–dorsal infundibulum (Pearson et al., 2011; Pearson and Placzek, 2013). Since the pattern of proliferation in the forming infundibulum is not homogeneous, we should examine not only sagittal sections but also transverse sections. However, our model for nurohypophysis development in mice is considered to be consistent with the model in the chick embryos, although further investigations are necessary.

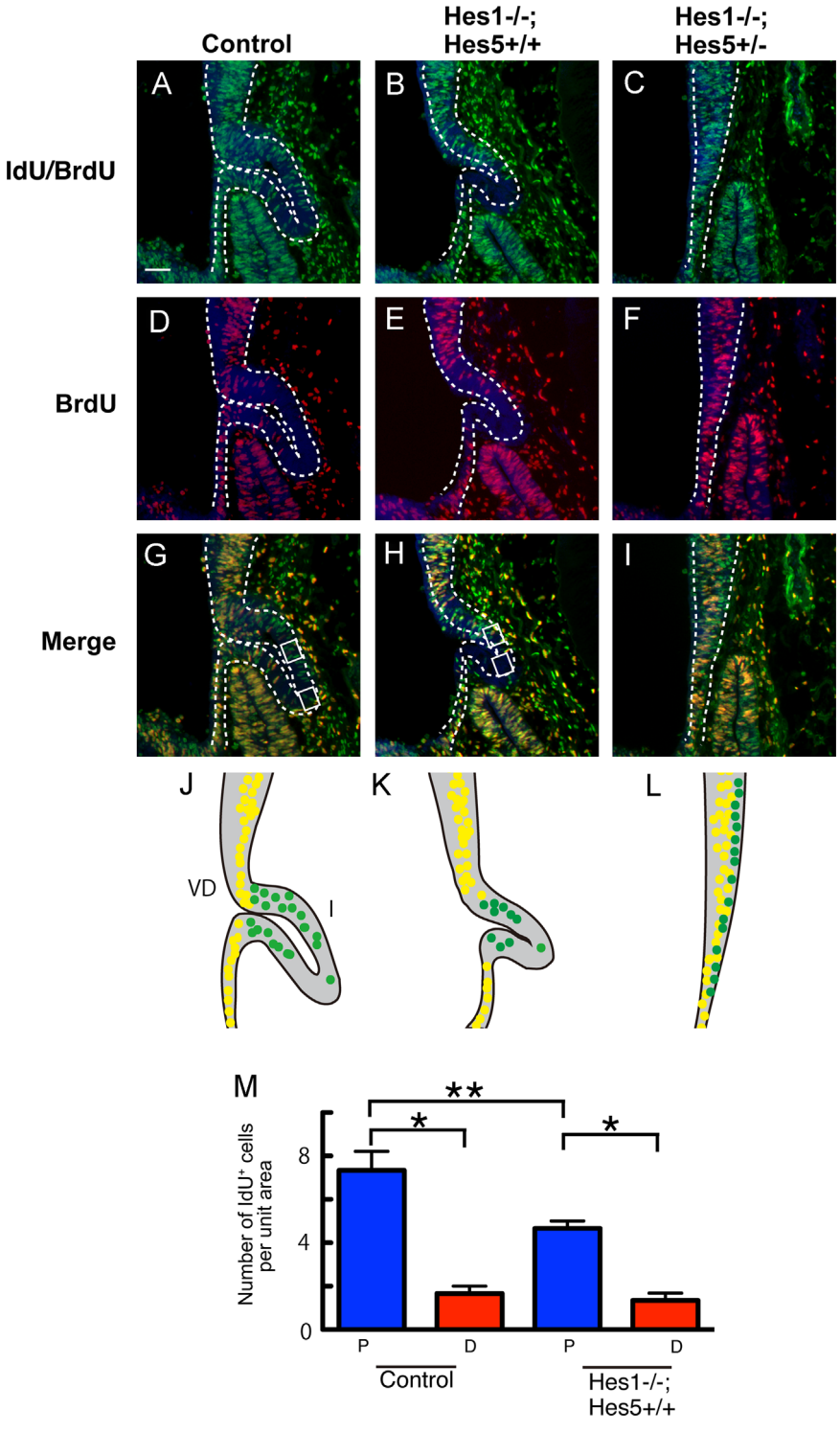
During limb development, the apical ectodermal ridge (AER), which is a specialized ectodermal structure formed at the distal tip of the limb bud, control outgrowth and pattern-

Fig. 5 – Progenitor cells differentiate into pituicytes at the base of the infundibulum. (A–L) Pulse chase labeling experiment with IdU and BrdU. (A–I) Immunostaining with anti-IdU and anti-BrdU antibodies. (J–L) Schematic representation of this experiment. We labeled cells at E11.5 by IdU and at E12.5 by BrdU. The anti-IdU antibody detects both IdU and BrdU, but the anti-BrdU antibody detects only BrdU. Therefore, cells that are labeled at E11.5 with IdU and at E12.5 with BrdU appear *green* when visualized with the anti-IdU antibody and Alexa 488 fluorescence (A–C). Cells that are labeled at E12.5 with BrdU appear *red* when visualized with the anti-BrdU antibody and Alexa 594 fluorescence (D–F). When these images are overlaid, the BrdU-labeled cells appear *yellow* as a result of the combination of the *red* and *green* fluorescence, and the IdU-labeled cells appear *green* (G–L). (A, D, G and J) In control mice of E12.5, BrdU-incorporated cells (*yellow*) were located in the ventral diencephalon, but not in the infundibulum. Cells that incorporated IdU at E11.5 (*green*) were located in the proximal portion of the infundibulum. (B, E, H, and K) In *Hes1*-mutant embryos, the evagination of the ventral diencephalon was observed but small compared with the control. Cells that incorporated IdU at E11.5 (*green*) were located in the proximal portion of the infundibulum but were significantly decreased compared with the control. (C, F, I, and L) In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the evagination was completely lost. IdU-incorporated cells were located in the outer layer of the infundibulum. (M) Quantification of IdU-incorporated cells. IdU^{+} cells were counted in the unit area (boxed areas in (G) and (H)). Three independent embryos of each genotype were examined. IdU^{+} cells were significantly decreased in the distal infundibulum compared to the proximal portion in both control and *Hes1*-null mice (*$p<0.005$, *t*-test). Moreover, IdU^{+} cells were significantly decreased in the proximal infundibulum of *Hes1*-null embryos compared with the control ($p<0.05$, *t*-test). VD, ventral diencephalon; I, infundibulum; P, proximal; D, distal. Bars, 50 μm (A–I).**

ing of the proximodistal limb–bud axis (Benazet and Zeller, 2009; Yu and Ornitz, 2008). By contrast, in neurohypophysis development, there is no structure like AER at the distal tip of the infundibulum. Our data show that proliferative zone is located at the base of the infundibulum but not in its distal tip. In neurohypophysis development, adjacent Rathke's pouch, which has close physical contact with the ventral diencephalon, may play a role in formation of the infundibulum like AER.

3.3. *Interaction between Rathke's pouch and the ventral diencephalon during pituitary development*

It is well described that signaling molecules expressed in the ventral diencephalon provides instructive cues for cell proliferation to form the pituitary gland. It has been reported that persistent Notch activation in the developing hypothalamus affects the development of the adenohypophysis (Aujla et al., 2015). The mutual interaction between Rathke's pouch



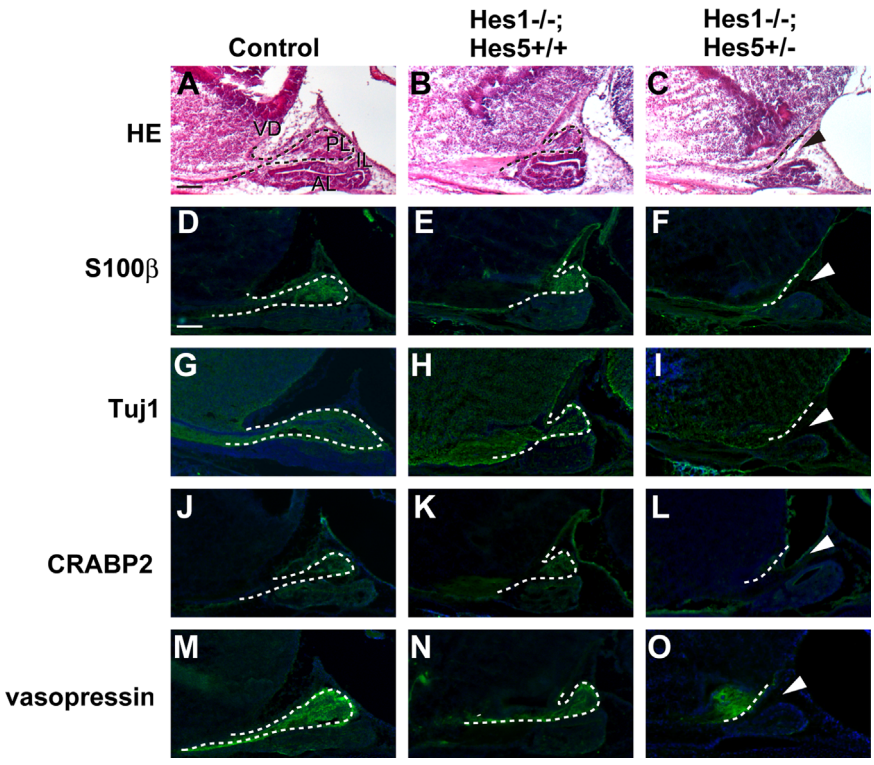


Fig. 6 – The neurohypophysis is lost in *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos at E16.5. (A–C) Midsagittal sections of E16.5 embryos were stained with HE. (A and B) In *Hes1*-mutant embryos, the posterior pituitary lobe was reduced in size but morphologically normal compared with the control (dashed lines). (C) In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the posterior pituitary lobe was completely lost (arrowhead). (D–F) Immunostaining with an anti-S100β antibody. (D and E) In both *Hes1*-null and control embryos, S100β^{+} cells (pituicytes) were observed in the posterior pituitary lobe (dashed line). (F) In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the posterior lobe was completely lost. (arrowhead). (G–I) Immunostaining with an anti-Tuj1 antibody. (G and H) In both *Hes1*-null and control embryos, Tuj1 was positive continuously from the ventral diencephalon to the posterior lobe (dashed lines). (I) In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the posterior lobe was completely lost (arrowhead). (J–L) Immunostaining with an anti-CRABP2 antibody. (J and K) In both *Hes1*-null and control embryos, CRABP2^{+} cells (pituicytes) were observed in the posterior pituitary lobe (dashed line). (L) In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the posterior pituitary lobe was completely lost (arrowhead). (M–O) Immunostaining with an anti-vasopressin antibody. (M and N) In both *Hes1*-null and control embryos, vasopressin was positive continuously from the ventral diencephalon to the posterior lobe (dashed lines). But, in *Hes1*-null embryos, the amount of vasopressin was reduced compared with the control. (O) In *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mutant embryos, the posterior pituitary lobe and the stalk were completely lost (arrowhead), and vasopressin was detected only in the ventral diencephalon. VD, ventral diencephalon; AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe. Bars, 50 μm (A–C and D–O).

and the ventral diencephalon has an essential role in the development of both anterior and posterior lobes. We have previously reported that the adenohypophysis is severely hypoplastic and dysmorphic in *Hes*-mutant mice (Kita et al., 2007). Therefore, the phenotype of the neurohypophysis, which is observed in this study, may be due in part to indirect signaling *via* the adenohypophysis, and further investigations are necessary.

3.4. *Other transcription factors and signaling pathways in neurohypophysis development*

The LIM-homeobox transcription factor Lhx2 is expressed in the developing ventral diencephalon (Zhao et al., 2010). In *Lhx2*-null embryos, the neuroectoderm of the ventral diencephalon fails to evaginate resulting in loss of the infundibulum. Instead of leaving the cell cycle, cells in this region continue to proliferate. The defect in formation of the infundibulum may result from an impairment of the regulation of proper cell cycle exit. By contrast, in the absence of *Hes* genes, progenitors prematurely exit from the cell cycle resulting in loss of the infundibulum. In these mutant mice, the phenotype of the neurohypophysis is expressed through completely different mechanisms. The T-box transcription factor Tbx3 is also expressed in the ventral diencephalon (Trowe et al., 2013). In *Tbx3*-deficient embryos, the ventral diencephalon is hyperproliferative, and displays an abnormal cellular architecture. The ventral diencephalon adjacent to Rathke's pouch is thickened and does not evaginate. These mutant mice are embryonic lethal and cannot be examined after E14.5. *Tbx3*-null embryos lack the infundibulum probably in the same mechanism as *Lhx2*-deficient mice, but not as *Hes*-mutants.

The HMG-box transcription factor SOX2 and SOX3 are expressed in neural precursors during neural development. They are also expressed in the ventral diencephalon during

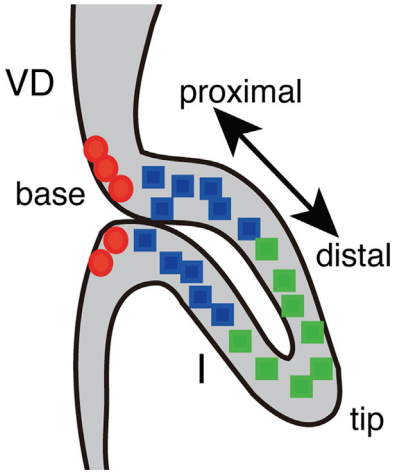


Fig. 7 – Schematic drawing showing the mechanism of neurohypophysis development. Progenitor cells of pituicytes (Hes1$^+$, Ki67$^+$, BrdU$^+$, calbindin$^-$, and S100β$^-$) are located at the base of the infundibulum (red dots). This area may be the proliferative zone. In this proliferative zone, progenitor cells are maintained and differentiated into pituicytes. Immature pituicytes are Hes1$^-$, Ki67$^-$, BrdU$^-$, calbindin^{low}, and S100β$^-$ (blue squares), and mature pituicytes are Hes1$^-$, Ki67$^-$, BrdU$^-$, calbindin$^-$, and S100β$^+$ (green squares). VD, ventral diencephalon; I, infundibulum.

pituitary development (Rizzoti, 2015). They maintain pluripotency and self-renewal potential in embryonic stem cells. It has been reported that *SOX2* haploinsufficient humans exhibit diencephalic abnormalities, such as hypothalamic hamartoma, an overgrowth of the hypothalamus (Kelberman et al., 2006). In a mouse model of human SOX2 haploinsufficiency, SOX2 hypomorphism disrupts the development of the hypothalamus, resulting in an ectopic protuberance of the prechordal floor and abnormal hypothalamic patterning (Langer et al., 2012). In this model mouse, the infundibulum is broadened, but the mechanism of this phenotype is not examined in detail (Langer et al., 2012). In humans, mutations in *SOX3* are associated with X-linked mental retardation and GH deficiency (Laumonnier et al., 2002). In *SOX3*-deficient mice, the evagination of the ventral diencephalon is less pronounced (Rizzoti et al., 2004). In the area of the ventral diencephalon where *SOX3* expression is reduced, cell proliferation is diminished. However in these mutant mice, development of the neurohypophysis is not examined. In our study, in the absence of *Hes1* and *Hes5*, progenitor cells were prematurely differentiated into neurons in the ventral diencephalon. Although the ventral diencephalon was hypertrophic, an overgrowth such as hamartoma was not observed in *Hes*-mutant mice.

During pituitary development, *Fgf8*, *Fgf10* and *Fgf18* are expressed in the ventral diencephalon (Rizzoti, 2015). In *Fgf10*-null mice, Rathke's pouch is absent and the development of the posterior lobe is arrested by E15.5 (Ohuchi et al., 2000). *Fgf10* is expressed in the infundibulum in wild-type mice at E11.5. The levels and boundaries of *Fgf10* expression are unchanged in *Hes1*-null mice (Raetzman et al., 2007). Similar results are also reported on *Fgf8* expression (Raetzman et al., 2007). Therefore, the phenotype of the neurohypophysis in *Hes*-mutants, which was observed in our study, may be independent with the FGF signaling pathways, although further investigations are necessary.

In neurohypophysis development, correlations between the Notch-*Hes* pathway and other transcription factors such as Lhx2, Tbx3, SOX2, and SOX3 remain to be elucidated. Although further studies are necessary, cross-talk between *Hes* genes and other factors must play crucial roles during neurohypophysis development.

4. Experimental procedures

4.1. *Mice*

All animals used in this study were maintained and handled according to protocols approved by Kyoto University. Genotypes of *Hes1* and *Hes5* mutant mice were determined as previously described (Cau et al., 2000; Kita et al., 2007; Kitagawa et al., 2013; Ohtsuka et al., 1999). *Hes1*$^{-/-}$; *Hes5*$^{+/-}$ mice were obtained by crossbreeding. All analyses were performed between littermates. At least three independent littermates were examined in each experiment.

4.2. *In situ hybridization*

Digoxigenin-labeled antisense RNA probes corresponding to fragments of *Hes1* and *Hes5* cDNAs were synthesized *in vitro*. *In situ* hybridization was performed as previously described (Hirata et al., 2001; Kita et al., 2007). Briefly, 16 μm-thick cryosections were treated with proteinase K, refixed with 0.2% glutaraldehyde and 4% paraformaldehyde, washed with 0.1% Tween 20 in PBS, and hybridized with RNA probe in 50% formamide, 5 × SSC, 1% SDS, 50 μg/ml heparin, and 50 μg/ml tRNA solution at 65 °C overnight. After hybridization, sections were washed at 65 °C in 50% formamide, 5 × SSC, 1% SDS, treated with ribonuclease, washed in 50% formamide, and 2x SSC, washed in Tris-buffered saline, and incubated with an alkaline-phosphatase-conjugated antibody against digoxigenin at 4 °C overnight. After incubation, the sections were washed with 0.1% Tween 20 in Tris-buffered saline three times, and in 100 mM NaCl, 100 mM Tris–HCl, pH 9.5, 50 mM $MgCl_2$, and 0.1% Tween 20 solution once. For a color development reaction, 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were used. Sections were analyzed with a Carl Zeiss Axiophoto microscope.

4.3. *Immunostaining*

Immunostaining was performed with the following antibodies: mouse anti-Ki67 (1:100; BD PharMingen), mouse anti-S100β (1:500; Sigma), mouse anti-Tuj1 (1:500; Covance), rabbit anti-calbindin (1:1000; Chemicon), rabbit anti-SOX2 (1:2000; Abcam), rabbit anti-CRABP2 (1:200; Protein Tech), and rabbit anti-vasopressin (1:1000; Chemicon) antibodies. Cryosections were incubated in 5% normal goat serum and 0.1% Triton X-100 at room temperature for 1 h, then incubated with primary antibodies at 4 °C overnight. Donkey or goat anti-species IgG conjugated with Alexa 488 or Alexa 594 (Molecular

Probes) was used for a secondary antibody. Samples were then treated with DAPI. Sections were analyzed with LSM510 confocal microscopy (Carl Zeiss). TUNEL assay was performed with a detection kit as indicated in the protocol provided by a manufacturer (Roche).

4.4. *Pulse chase experiment*

For the IdU/BrdU pulse chase experiment, pregnant mice were injected intraperitoneally with IdU at E11.5 at 0.1 mg/g body weight and then injected intraperitoneally with BrdU at E12.5 at 0.1 mg/g body weight (Ward et al., 2005). One hour later, embryos were harvested. After antigen retrieval with 2 N HCl at 37 °C for 10 min, BrdU was detected with a rat anti-BrdU antibody (1:500; AbD Serotec) and a goat anti-rat IgG antibody conjugated with Alexa 594 (Molecular Probes). IdU was detected with a mouse anti-IdU/BrdU antibody (1:200; BD Biosciences) and a goat anti-mouse IgG antibody conjugated with Alexa 488 (Molecular Probes). Samples were then treated with DAPI. Sections were analyzed with LSM510 confocal microscopy (Carl Zeiss). At least three independent littermates were examined in each experiment.

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