The proneural bHLH genes *Mash1*, *Math3* and *NeuroD* are required for pituitary development

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Abstract

Multiple signaling molecules and transcription factors are required for pituitary development. Activator-type bHLH genes *Mash1*, *Math*, *NeuroD* (*Neurod*) and Neurogenin (*Neurog*) are well known as key molecules in neural development. Although analyses of targeted mouse mutants have demonstrated involvement of these bHLH genes in pituitary development, studies with single-mutant mice could not elucidate their exact functions, because they cooperatively function and compensate each other. The aim of this study was to elucidate the roles of *Mash1*, *Math3* and *NeuroD* in pituitary development. *Mash1;Math3;NeuroD* triple-mutant mice were analyzed by immunohistochemistry and quantitative real-time RT-PCR. Misexpression studies with retroviruses in pituisphere cultures were also performed. The triple-mutant adenohypophysis was morphologically normal, though the lumen of the neurohypophysis remained unclosed. However, in triple-mutant pituitaries, somatotropes, gonadotropes and corticotropes were severely decreased, whereas lactotropes were increased. Misexpression of *Mash1* alone with retrovirus could not induce generation of hormonal cells, though *Mash1* was involved in differentiation of pituitary progenitor cells. These data suggest that *Mash1*, *Math3* and *NeuroD* cooperatively control the timing of pituitary progenitor cell differentiation and that they are also required for subtype specification of pituitary hormonal cells. *Mash1* is necessary for corticotroph and gonadotroph differentiation, and compensated by *Math3* and *NeuroD*. *Math3* is necessary for somatotroph differentiation, and compensated by *Mash1* and *NeuroD*. *Neurog2* may compensate *Mash1*, *Math3* and *NeuroD* during pituitary development. Furthermore, *Mash1*, *Math3* and *NeuroD* are required for neurohypophysis development. Thus, *Mash1*, *Math3* and *NeuroD* are required for pituitary development, and compensate each other.

Key Words

- *Mash1*
- *Math3*
- *NeuroD*
- pituitary development
Introduction

The pituitary gland is composed of two distinct entities: the adenohypophysis and the neurohypophysis. The formation of the primordium of the adenohypophysis is controlled by signaling molecules from the ventral diencephalon. In addition to signaling molecules, various transcription factors play roles in the formation of Rathke's pouch and the generation of endocrine cell lineages in the adenohypophysis (Davis et al. 2013, Rizzoti 2015). Prophet of Pit1 (Prop1) is critical for initial activation of Pit1-lineages: thyrotropes, somatotropes and lactotropes (Ward et al. 2005). Recently, it has been reported that all types of adenohypophyseal cells derived from Prop1-expressing progenitors (Davis et al. 2016). Pit1 is required for terminal differentiation of endocrine cells such as thyrotropes, somatotropes and lactotropes (Lin et al. 1994). Differentiation of corticotropes is dependent on Tpit/Tbx19 (Liu et al. 2001, Lamotte et al. 2004).

In many organs, cell proliferation and differentiation are regulated by multiple basic helix-loop-helix (bHLH) genes. The repressor-type bHLH genes include Hes genes, homologs of Drosophila hairy and Enhancer of split (E(spl)) (Kageyama et al. 2007). The activator-type bHLH genes include Mash1/Ascl1, Math, NeuroD (Neurog) and Neurogenin (Ngf/Neurog), homologs of Drosophila proneural genes achate-scute complex and atonal (Kageyama et al. 2005). It has been reported that Hes1 and Hes5 control adenohypophyseal progenitor cells during pituitary development (Zhu et al. 2006, Kita et al. 2007, Raetzman et al. 2007). Furthermore, Hes1 and Hes5 are essential for formation of the neurohypophysis (Goto et al. 2015).

Activator-type bHLH genes are well known as key molecules in the development of the nervous system and digestive organs (Kageyama & Nakanishi 1997). These factors are also involved in pituitary development. NeuroD is initially detected at embryonic day 11.5 (E11.5) in the rostoventral region of Rathke's pouch (Liu et al. 2001). NeuroD is involved in corticotroph development, but the phenotype of NeuroD single-mutant pituitaries is very faint (Poulin et al. 1997, Liu et al. 2001, Lamotte et al. 2004). Mash1 is initially detected in the evaginating oral ectoderm at E9.5 (Liu et al. 2001). It has been reported that POMC expression appears unaltered in Mash1-mutant pituitaries (McNay et al. 2006). However, detailed analysis has revealed that POMC expression is decreased in Mash1-mutant pituitaries (Zhang et al. 2015). Math3 expression is initially observed at E13.5 in the presumptive anterior lobe and persists in the anterior lobe of the adult pituitary.

In Math3-mutant embryos, the number of somatotropes is decreased (Zhu et al. 2006). Ngn2 is expressed weakly in Rathke's pouch at E12.5. However, in Ngn1;Ngn2 double-mutant mice, the pituitary gland is formed normally (Lamotte et al. 2004). It is well known that these bHLH genes compensate each other and cooperatively regulate neural development in mice (Akagi et al. 2004). In pituitary development, bHLH genes may cooperatively regulate hormonal cell development. The above-mentioned weak phenotypes of mutant mice may be due to compensation by other bHLH genes. Therefore, we decided to analyze Mash1;Math3;NeuroD triple-mutant mice in order to elucidate exact roles of bHLH genes in pituitary development. Since the pituitary gland is so small that we cannot obtain enough protein from it for quantitative analyses, we performed immunohistochemistry and quantitative real-time RT-PCR. Here, we show that Mash1, Math3 and NeuroD cooperatively regulate the initiation of adenohypophyseal development and hormonal cell specification in pituitary development.

Materials and methods

Generation of double and triple mutant mice

All animals used in this study were maintained and handled according to protocols approved by Kyoto University. Math3-, Mash1- and NeuroD-mutant mice were generated previously (Guillemot et al. 1993, Miyata et al. 1999, Tomita et al. 2000). Math3;NeuroD double mutant mice were obtained by crossing double heterozygous mice. Mash1;Math3;NeuroD triple mutant mice were obtained by crossing triple heterozygous mice or triple-heterozygous female and Mash1+/−;NeuroD+/−;Math3−/− male mice. Their genetic background was ICR. NeuroD-mutant mice die shortly after birth due to severe diabetes (Naya et al. 1997). Mash1-mutant mice die in the first 24 h after birth, though the cause is unknown (Guillemot et al. 1993). Therefore, the mutant embryos were obtained at E17.5.

Immunostaining

Immunostaining was performed with the following antibodies as previously described: rabbit anti-POMC (ACTH) (1:4000; Chemicon), rabbit anti-GH (1:4000; Chemicon), rabbit anti-PRL (1:1000; Chemicon), mouse anti-TSH (1:50; Dako), mouse anti-FSH (1:50; Dako), mouse anti-LH (1:50; Dako), mouse anti-Mash1 (1:500; BD Pharmingen), goat anti-NeuroD (1:200; Santa Cruz), rabbit anti-SOX2 (1:2000; Abcam), rabbit anti-GFP.
(1:500; Invitrogen) and rat anti-GFP (1:200; Nacalai Tesque) antibodies (Kita et al. 2007, Imayoshi et al. 2013, Kitagawa et al. 2013). Briefly, cryosections were incubated in 5% normal goat serum and 0.1% Triton X-100 at room temperature for 1 h, and 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).

Quantitative real-time RT-PCR

Total RNA samples were extracted from the pituitary glands of embryos at E17.5 using TRIzol reagent and the RNeasy Mini Kit (Qiagen). RNA samples were subjected to reverse transcription, and real-time RT-PCR was performed in duplicate by using Applied Biosystems 7500 Real Time PCR System (Applied Biosystems) and Thunderbird SYBR qPCR Mix (TOYOBO) according to the manufacturer's protocols, as previously described (Ohtsuka et al. 2011, Tan et al. 2012, Watanabe et al. 2015). Primers were designed using Primer-BLAST (Ye et al. 2012) or previously reported sequences (Shima et al. 2008, Suga et al. 2011) were used (Table 1). At least 3 independent embryos were examined for each genotype. Values of mRNA expression of each gene were normalized by the values of GAPDH.

Table 1 Primers used for quantitative real-time RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’ to 3’)</th>
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<tbody>
<tr>
<td>Mash1</td>
<td>F: TACCTTTCGCAGGGCACTTC</td>
</tr>
<tr>
<td></td>
<td>R: GCGAAAGGAGCAAGGTTGTT</td>
</tr>
<tr>
<td>NeuroD</td>
<td>F: CGAGGTCCTCGAGGATAGGT</td>
</tr>
<tr>
<td></td>
<td>R: CCGCCCTCTGCAGGTATGTT</td>
</tr>
<tr>
<td>Neurog2</td>
<td>F: TCGGGTTTAACTGGAGTGCC</td>
</tr>
<tr>
<td></td>
<td>R: GTGTTGTGCTGTTCTGTGTC</td>
</tr>
<tr>
<td>Prl</td>
<td>F: GCTGTTTCGCAAAATGTTCAGC</td>
</tr>
<tr>
<td></td>
<td>R: GGTCTTGACATACCTTTATGCAA</td>
</tr>
<tr>
<td>Gh</td>
<td>F: GCTAGTGCTTTTCGCCCAT</td>
</tr>
<tr>
<td></td>
<td>R: GTAGGGAGGATGAGACAGG</td>
</tr>
<tr>
<td>Tshb</td>
<td>F: TGGTTATGTATGACAGG</td>
</tr>
<tr>
<td></td>
<td>R: GACCTCGTGGATTTCCACCG</td>
</tr>
<tr>
<td>Pomp</td>
<td>F: GCCGTCCCTCTAGAGTTCA</td>
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<tr>
<td></td>
<td>R: GACGTGCTCAAGCCAAAAATG</td>
</tr>
<tr>
<td>Lhb</td>
<td>F: CTGGTCAAGGCACTCTGGC</td>
</tr>
<tr>
<td></td>
<td>R: CAGTACTGGCAGTGTTAGGAGC</td>
</tr>
<tr>
<td>Fshb</td>
<td>F: TCTGGTGCTGGAGAGCAATC</td>
</tr>
<tr>
<td></td>
<td>R: GCGAGCTGGGCTTATAC</td>
</tr>
<tr>
<td>Pit1</td>
<td>F: GAGAGGTTGGAGCAAGAGGA</td>
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<tr>
<td></td>
<td>R: TCGGCCATGGCAGCATG</td>
</tr>
<tr>
<td>SF1 (Nr5a1)</td>
<td>F: GTAGGGCCAAAGGAGGACAGCAT</td>
</tr>
<tr>
<td></td>
<td>R: CCACAGGCACAAATAGCAACTG</td>
</tr>
<tr>
<td>Gapdh</td>
<td>F: GGAACCCTAGGGCCTACATG</td>
</tr>
<tr>
<td></td>
<td>R: TAGGGCTCTTGTGCTCAGTG</td>
</tr>
</tbody>
</table>

Pituitispher culture

Isolation of pituitary cells and pituisphere cultures were performed as previously described (Chen et al. 2005, Fauquier et al. 2008). Briefly, anterior pituitary lobes were obtained from 7-week-old ICR mice, and dissociated into single cells by using trypsin (2.5%; 15 min). Freshly dissociated cells were seeded in DMEM/F12 containing 0.5% BSA, B27 (Gibco) and N2 (R&D systems) supplements plus 20 ng/ml bFGF and EGF (Invitrogen). After 4 days of culture, virus solution was applied with polybrene (4 µg/mL). Three days after infection, pituispheres were collected and plated on Matrigel-coated eight-well glass slides in DMEM/F12 containing FCS (10%), horse serum (10%), B27 and N2 supplements. After 2 days of culture, pituispheres were fixed with 4% paraformaldehyde.

Construction of retroviruses

For pMX-EGFP-Mash1 and pMX-EGFP-Math3, the bHLH genes fused with EGF were cloned into the EcoRI and XhoI sites of pMXs (Cell Biolabs) (Kitamura 1998, Hatakeyama et al. 2001, Inoue et al. 2002). Retroviral DNAs were transfected with Lipofectamine (Gibco-BRL) into Plat-E (Cell Biolabs), an ecotropic packaging cell line (Morita et al. 2000, Kim et al. 2007). The supernatant was collected 2 days later and concentrated with Centricron Plus-20 (Millipore), as described previously (Bae et al. 2000, Hojo et al. 2000). Ten microliters of the virus solution was applied to the pituisphere cultures.

Results

**Mash1 and NeuroD expression is increased in double-mutant pituitaries**

It has been reported that Mash1, Math3 and NeuroD are expressed in the developing pituitary gland (Poulin et al. 1997, Liu et al. 2001, Lamote et al. 2004, Zhu et al. 2006, Zhang et al. 2015). Math3 expression persists in the anterior lobe until E17.5, while Mash1 expression is more intensively sustained in the intermediate lobe. However, NeuroD is scarcely detected in the anterior lobe,
and never expressed in the intermediate lobe at E17.5. It is also well known that these bHLH genes cooperatively specify neuronal subtypes during neural development (Akagi et al. 2004). Therefore, it is likely that these bHLH genes may compensate each other and cooperatively play roles in pituitary development. To test this possibility, we analyzed Mash1 and NeuroD mRNA levels in double-mutant pituitaries by quantitative RT-PCR. Expression of Mash1 was significantly upregulated in Math3; NeuroD double-mutant pituitaries compared with the control (P<0.02, Fig. 1A), suggesting that Mash1 may compensate Math3 and NeuroD during pituitary development. Expression of NeuroD was upregulated by about 10% in Mash1; Math3 double-mutant pituitaries compared with the control, although not statistically significant (data not shown). Therefore, we analyzed NeuroD expression in Mash1; Math3 double-mutant pituitaries by immunohistochemistry. Expression of NeuroD was detected in the intermediate lobe, and increased in the anterior lobe compared with the control (Fig. 1B and C), suggesting that NeuroD may compensate Mash1 and Math3 during pituitary development. Thus, to understand the roles of these bHLH factors in pituitary development, we decided to analyze Mash1; Math3; NeuroD triple-mutant embryos.

**Delayed differentiation of adenohypophyseal progenitor cells in Mash1;Math3;NeuroD triple-mutant embryos**

We first examined the morphology and size of Mash1;Math3;NeuroD triple-mutant pituitaries at E17.5. The mutant adenohypophysis was morphologically normal, but the lumen of the neurohypophysis remained unclosed (Fig. 2A and B). To examine whether apoptosis was responsible for this phenotype, we performed TUNEL assay. In Mash1; Math3; NeuroD triple-mutant pituitaries, TUNEL+ cells were not significantly increased compared with the control (Fig. 2C and D), suggesting that this phenotype is not due to apoptosis.

To investigate the degree of differentiation of progenitor cells, we examined expression of SOX2, a marker of pituitary progenitors, in mutant pituitaries. SOX2 is a member of the SOXB1 subfamily of HMG box transcription factors and is required for the maintenance of stem cells. In the periluminal region of triple-mutant anterior pituitary lobes, Sox2+ cells were increased compared with the control (Fig. 2E and F, bracket). Insm1 is a zinc-finger transcription factor that regulates the maturation of progenitor cells in the developing central nervous system. It has been reported that Insm1 is a direct target of Mash1 (Jacob et al. 2009). In Insm1-mutant mice, Sox2- and Sox9-positive cells were increased in the periluminal area, especially in the caudal region, of anterior pituitary lobes (Welcker et al. 2013). This phenotype closely resembles that of our Mash1; Math3; NeuroD triple-mutant embryos. It is considered that this similarity is supportive of our results on Sox2 immunostaining. Therefore, Sox2+ cells were counted in the caudal area of anterior pituitary lobes. The number of Sox2+ cells and the total number of cells in this region (100 μm x 50 μm) were counted, and the ratio of Sox2+ cells was calculated. Three independent embryos of each

![Figure 1](https://doi.org/10.1530/JME-18-0090)
Proneural bHLH genes in pituitary development

Mash1, Math3 and NeuroD are required for subtype specification of pituitary endocrine cells

To determine whether bHLH factors can control subtype specification of endocrine cells during pituitary development, we analyzed cell lineages including somatotropes, lactotropes, thyrotropes, corticotropes and gonadotropes. First, we examined somatotropes (GH-secreting cells) in mutant pituitaries by immunohistochemistry and quantitative real-time RT-PCR (Fig. 3A, B, C, D and E), because it has been previously reported that Math3 is required for the proper initiation of somatotrope specification (Zhu et al. 2006). At E17.5, GH+ cells were significantly decreased in Math3-null pituitaries compared with the control (P<0.0001, Fig. 3A, B and D). The number of GH+ cells was counted in each unit area (100 µm x 50 µm) on the rostral side, the middle side and the caudal side of anterior pituitaries. The number of GH+ cells in these three areas was summed up as the number of GH+ cells. At least three independent embryos were examined for each genotype. Strikingly, GH+ cells were more prominently decreased and scarcely detected in Mash1; Math3; NeuroD triple-mutant pituitaries (P<0.0001, Fig. 3A, C and D). GH mRNA levels were also significantly reduced in Math3 single-mutant pituitaries compared with the control (P<0.002, Fig. 3E). GH mRNA levels were further reduced in Mash1; Math3; NeuroD triple-mutant pituitaries compared with Math3 single-mutant embryos (P<0.005, Fig. 3E). Since somatotropes are Pit1-lineage cells, we examined Pit1 mRNA levels in Mash1; Math3; NeuroD triple-mutant pituitaries. In Mash1; Math3; NeuroD triple-mutant mice, Pit1 mRNA levels were significantly upregulated compared with the control (P<0.02, Fig. 3F). These results suggest that Math3, Mash1 and NeuroD cooperatively function downstream of Pit1 and are required for specification of GH-secreting cells. Next, we examined the other Pit1-lineage cells, lactotropes (PRL-secreting cells) and thyrotropes (TSH-secreting cells), by quantitative real-time RT-PCR and immunohistochemistry. PRL mRNA levels were also significantly increased in Math3 single-mutant pituitaries compared with the control (P<0.05, Fig. 3G). In Mash1; Math3; NeuroD triple-mutant embryos, PRL mRNA levels were further upregulated (P<0.05, Fig. 3G). PRL+ cells were increased in Math3-null pituitaries compared with the control (open arrowheads, Fig. 3H and I). PRL+ cells were more prominently increased in Mash1; Math3 double-mutant pituitaries (open arrowheads, Fig. 3H and J). However, in Math3 single-mutant and Mash1; Math3; NeuroD triple-mutant embryos, TSH mRNA levels were not changed compared to the control (Fig. 3K). TSH+ cells were also not changed in Math3 single-mutant and Mash1; Math3; NeuroD triple-mutant pituitaries (Fig. 3L, M and N). These data suggest that Pit1+ precursors differentiate into PRL-secreting cells at the expense of GH-secreting cells in the absence of Mash1, Math3 and NeuroD.

Figure 2

Delayed pituitary differentiation in Mash1; Math3; NeuroD triple-mutant embryos. The mutant adenohypophysis was morphologically normal, but the lumen of the neurohypophysis remained unclosed (A and B). TUNEL+ cells were not significantly increased (C and D). In the mutant anterior pituitary lobes, Sox2+ cells were increased compared with the control (E and F, bracket). The proportions of Sox2+ cells were significantly increased in triple-mutant pituitaries compared with the control (G). n=3 (control), and 3 (triple mutants). A, anterior lobe; I, intermediate lobe; P, posterior lobe; TKO, triple knockout. Bar, 50 µm (A, B, C, D, E and F). A full colour version of this figure is available at https://doi.org/10.1530/JME-18-0090.
Next, we examined corticotropes (POMC-producing cells) in mutant pituitaries by immunohistochemistry and quantitative real-time RT-PCR (Fig. 4). Previously, it has been reported that NeuroD is involved in the development of corticotropes, but the phenotype of NeuroD single-mutant mice is very faint (Liu et al. 2001, Lamolet et al. 2004). It has also been reported that expression of POMC is decreased but observed in Mash1-null pituitaries (Zhang et al. 2015). It is likely that other bHLH factors compensate for loss of NeuroD and Mash1 in corticotrope development. At E17.5, POMC+ cells were significantly decreased in Mash1-null pituitaries compared with the control (P < 0.005, Fig. 4A, B and D). POMC+ cells were counted in the same manner as for GH+ cells. POMC+ cells were more prominently decreased in Mash1; Math3; NeuroD triple-mutant pituitaries (P < 0.005, Fig. 4A, B and D). In Mash1 single-mutant pituitaries, POMC mRNA levels were downregulated, although not statistically significant (Fig. 4E). In Mash1; Math3; NeuroD triple-mutant mice, POMC mRNA levels were significantly downregulated compared with the control (P < 0.05), though there was no difference when compared to Mash1 single-mutant pituitaries (Fig. 4E). These results suggest that Mash1, NeuroD and Math3 cooperatively control corticotrope specification in pituitary development.

Finally, we examined gonadotropes (FSH-secreting and LH-secreting cells) in mutant pituitaries by immunohistochemistry and quantitative real-time RT-PCR (Fig. 5A, B, C, D, E, F, G and H). At E17.5, FSH+ cells were significantly decreased in Mash1; Math3; NeuroD triple-mutant pituitaries compared to the control (P < 0.001, Fig. 5A, B and C). LH+ cells were also significantly decreased in Mash1; Math3; NeuroD triple-mutant pituitaries compared to the control (P < 0.05, Fig. 5D, E and F). The number of all FSH+ or LH+ cells in each section was counted. FSH and LH mRNA levels were also significantly reduced in Mash1 single-mutant pituitaries compared with the control (P < 0.05, Fig. 5G

![Figure 3](http://jme.endocrinology-journals.org/sites/default/files/fig3.jpg)

**Figure 3**
Math3 is required for Pit1-lineage differentiation and compensated by Mash1 and NeuroD. At E17.5, GH+ cells were decreased in Math3-null pituitaries compared with the control (A and B). GH+ cells were more prominently decreased and scarcely detected in Mash1; Math3; NeuroD triple-mutant pituitaries (C). Quantification of GH+ cells (D). GH+ cells were significantly decreased in Math3-null pituitaries compared with the control. GH+ cells were more prominently decreased in Mash1; Math3; NeuroD triple-mutant pituitaries (D). n = 8 (control), 5 (Math3 mutants), and 3 (triple mutants). GH mRNA levels were significantly reduced in Math3 single-mutant pituitaries compared with the control (E). GH mRNA levels were further reduced in Mash1; Math3; NeuroD triple-mutant pituitaries compared with Math3 single-mutant embryos (E). n = 6 (control), 5 (Math3 mutants), and 5 (triple mutants). In Mash1; Math3; NeuroD triple-mutant mice, Pit1 mRNA levels were significantly up-regulated compared with the control (F). n = 6 (control), 5 (Math3 mutants), and 5 (triple mutants). In Mash1; Math3; NeuroD triple-mutant embryos, PRL mRNA levels were significantly up-regulated (G). n = 5 (control), 3 (Math3 mutants), and 3 (triple mutants). PRL+ cells were increased in Math3-null pituitaries compared with the control (H and I, open arrowheads). PRL+ cells were more prominently increased in Mash1; Math3 double-mutant pituitaries (I, open arrowheads). In Mash1; Math3; NeuroD triple-mutant embryos, TSH mRNA levels were not changed compared with the control (K). n = 6 (control), 4 (Math3 mutants), and 4 (triple mutants). TSH+ cells were not changed in Mash3 single-mutant and Mash1; Math3; NeuroD triple-mutant pituitaries compared to the control (L, M and N). A, anterior lobe; I, intermediate lobe; TKO, triple knockout. Bar, 50 µm (A, B, C, H, I, J, L, M and N). A full colour version of this figure is available at https://doi.org/10.1530/JME-18-0090.
Mash1, Math3 and NeuroD cooperatively control gonadotrope differentiation. At E17.5, FSH+ cells were significantly decreased in Mash1; Math3; NeuroD triple-mutant pituitaries compared to the control (A, B and C). LH+ cells were also significantly decreased in Mash1; Math3; NeuroD triple-mutant pituitaries compared to the control (D, E and F, open arrows). n = 7 (control), 3 (Math3 mutants), and 5 (triple mutants). SF-1 mRNA levels were significantly increased in Mash1; Math3; NeuroD triple-mutant pituitaries compared with the control (G and H). FSH and LH mRNA levels were further reduced in Mash1; Math3; NeuroD triple-mutant pituitaries compared to the control (G and H). n = 7 (control), 3 (triple mutants). Neurog2 mRNA levels were significantly increased in Mash1; Math3; NeuroD triple-mutant pituitaries (I). n = 3 (control), and 3 (triple mutants). Bar, 50 µm (A, B and C). A full colour version of this figure is available at https://doi.org/10.1530/JME-18-0090.

Neurog2 may compensate for loss of Mash1, Math3 and NeuroD in pituitary development

Unlike in zash1a-mutant zebrafish, the pituitary gland appeared morphologically normal and did not disappear in Mash1 single-mutant mice. Even in Mash1; Math3;
In the developing retina, Neurog2, NeuroD, Mash1 and Math3 cross-regulate each other, and cooperatively specify neuronal subtypes (Akagi et al. 2004). Therefore, we examined Neurog2 expression in Mash1; Math3; NeuroD triple-mutant pituitaries. Strikingly, Neurog2 mRNA levels were significantly increased in triple-mutant pituitaries (P<0.05, Fig. 5J), suggesting that Neurog2 may compensate for loss of Mash1, Math3 and NeuroD in pituitary development.

Gain-of-function analysis of Mash1 and Math3 in adenohypophyseal development

In order to determine whether Mash1 and Math3 are sufficient for differentiation of pituitary hormonal cells, Mash1 and Math3 were misexpressed with retroviruses in pituisphere cultures (Fauquier et al. 2008). Since retroviruses are infectious only to mitotic cells, they are suitable for changing stably the phenotypes of progenitor cells. pMX-GFP, pMX-GFP-Mash1 and pMX-GFP- Math3 were used to generate retroviruses (Onishi et al. 1996, Hatakeyama & Kageyama 2002, Inoue et al. 2002). pMX-GFP-Mash1 directs expression of Mash1 fused with EGFP, and pMX-GFP-Math3 directs expression of Math3 fused with EGFP. All pMX-GFP-Mash1-infected cells coexpressed Mash1 and GFP (Fig. 6A, B, C and D). When the control virus pMX-GFP was applied, almost all virus-infected cells did not express POMC (Fig. 6E, F, G and H). When pMX-GFP-Mash1 was applied, almost all virus-infected cells did not also express POMC (Fig. 6I, J, K and L). Although a few virus-infected cells expressed POMC (Fig. 6M, N, O and P, arrowheads), pMX-GFP-Mash1 could not significantly induce POMC+ cells (Fig. 6I, J, K and L). When the control virus pMX-GFP was applied, almost all virus-infected cells strongly expressed Sox2 (Fig. 6Q, R, S and T). However, when pMX-GFP-Mash1 was applied, expression level of Sox2 was decreased in some of virus-infected cells (Fig. 6U, V, W and X, arrows). These results suggest that Mash1 may be involved in differentiation of pituitary stem cells, but is not sufficient for development of POMC-producing cells under this condition. When pMX-GFP-Math3 was applied, almost all virus-infected cells did not express GH (Fig. 7A, B, C and D). Although a few virus-infected cells expressed GH (Fig. 7E, F, G and H, arrowheads), pMX-GFP-Math3 could not significantly induce GH+ cells (Fig. 7A, B, C and D). These data suggest that Math3 alone is not sufficient for differentiation of GH-producing cells under this condition. Taken together, Mash1 or Math3 alone cannot promote pituitary hormonal cell genesis during adenohypophyseal development.

Discussion

Mash1, Math3 and NeuroD are required for initiation of pituitary endocrine cell differentiation

In Mash1; Math3; NeuroD triple-mutant pituitaries, Sox2+ cells were increased compared with the control, suggesting that these three bHLH factors cooperatively control the timing of endocrine progenitor cell differentiation during pituitary development. This function of Mash1 in mouse pituitary development is similar to that of zash1a in zebrafish development (Pogoda et al. 2006). In zash1a/asc1a (Mash1/Ascl1 homolog) mutant zebrafish embryos, adenohypophyseal cells fail to express hormone genes and display features of adenohypophyseal progenitors (Pogoda et al. 2006). Zash1a/asc1a is required for the initiation of pituitary specification and terminal differentiation of all adenohypophyseal cell types during zebrafish pituitary organogenesis. In the developing nervous system, the proneural gene Mash1 coordinates a genetic neuronal fate and a subtype specification (Imayoshi & Kageyama 2014). A similar proneural-like function of Mash1 may apply in adenohypophyseal development. In neural development, Notch-Hes signaling is essential for maintaining neural progenitor cells (Kageyama et al. 2015). Activation of Notch signaling leads to induction of Hes1 and Hes5. Hes1 and Hes5 directly repress expression of proneural genes, such as Mash1, Math, NeuroD and Neurogenin. Inactivation of Hes genes leads to upregulation of proneural genes and acceleration of neurogenesis. Antagonistic regulation between activator-type and repressor-type factors is important for maintenance of neural progenitor cells and proper timings of cell differentiation (Kageyama et al. 2008). It has been reported that Hes1 controls progenitor cells during adenohypophyseal development (Zhu et al. 2006, Kita et al. 2007, Raetzman et al. 2007). Notch ligands and receptors are expressed in early stages of pituitary development (Zhu et al. 2006). Hence, Notch-Hes signaling may regulate expression of Mash1, Math3 and NeuroD in adenohypophyseal development, like in neural development.

Math3 is required for Pit1 lineage differentiation and compensated by Mash1 and NeuroD

It has been reported that GH-, PRL- and TSH-producing cells are decreased in Pit1-mutant embryos (Li et al. 1990). In our study, Mash1; Math3; NeuroD triple-mutant pituitaries exhibited upregulation of Pit1, downregulation of GH and upregulation of PRL, while TSH expression...
was not affected. Previously, Zhu et al. have reported that in Math3 single-mutant embryos, Pit1, PRL and TSH expression levels were not changed, though GH was downregulated (Zhu et al. 2006). They have also reported that Math3 is a direct downstream target of Pit1 (Zhu et al. 2006). In our study, the decrease of GH+ cells was more prominent in Mash1; Math3; NeuroD triple-mutant embryos than in Math3 single-mutant mice. Our data suggest that Mash1 and NeuroD may compensate for loss of Math3 downstream of Pit1.

Lactotrope and somatotrope differentiation are completely dependent on Pit1 activation (de Moraes et al. 2012). These two cell types arise from the same precursors. Our data suggest the possibility that Pit1+ cells differentiated into PRL+ cells at the expense of GH+ cells in the absence of Mash1, Math3 and NeuroD. If Mash1,
Math3 and NeuroD are expressed, the Pit1+ progenitor cells may differentiate into GH+ cells. Whereas if Mash1, Math3 or NeuroD is not expressed, Pit1+ cells may differentiate into PRL+ cells. Math3 is necessary for the proper onset of somatotrope specification, and Mash1 and NeuroD may compensate for loss of Math3 in pituitary development.

**Mash1, Math3 and NeuroD are required for POMC lineage differentiation**

NeuroD can trigger corticotrope-specific transcription during pituitary development (Poulin et al. 1997). However, Liu et al. have reported that all cell types appear almost normal in NeuroD-null pituitaries (Liu et al. 2001). Lamolet et al. have reported that in NeuroD-null mice, corticotroph differentiation is delayed, but the delay is transient. NeuroD is required for corticotroph differentiation but not for commitment (Lamolet et al. 2004). NeuroD is initially expressed at E11.5 in the rostroventral region of Rathke’s pouch, but becomes scarcely detectable by E14.5 and is not expressed in the intermediate lobe (Liu et al. 2001). In intermediate lobe cells, this expression pattern is not supportive of a role of NeuroD in POMC expressing cells.

In contrast, Mash1 is expressed in the presumptive anterior lobe, and its expression is sustained in the intermediate lobe at a later stage in a pattern fully corresponding to POMC expressing cells (Liu et al. 2001). Recently, Zhang et al. have reported that POMC expression was decreased in Mash1-mutant pituitaries. Mash1 activates a large set of cell-type-specific enhancers during pituitary development (Zhang et al. 2015). However, POMC expression was not disappeared in Mash1 single-mutant pituitaries. It may be due to redundancy with other members of the bHLH family. It has been reported that the bHLH genes are functionally redundant for cell fate specification during the development of various tissue (Akagi et al. 2004). Therefore, we analyzed Mash1;Math3;NeuroD triple-mutant pituitaries. The expression of POMC was markedly decreased but still remained in triple-mutant pituitaries. Interestingly, Ngn2 mRNA levels were significantly increased in triple-mutant pituitaries. It is likely that Ngn2 may compensate for loss of Mash1, Math3 and NeuroD in corticotroph development.

**Mash1, Math3 and NeuroD control gonadotrope differentiation**

In Mash1;Math3;NeuroD triple-mutant pituitaries, gonadotropes were markedly decreased, and mRNA levels of FSH and LH were also extremely reduced. SF-1 has been characterized as a molecule required for differentiation of gonadotropes (Inghram et al. 1994). However, in our study, SF-1 was not affected in Mash1; Math3; NeuroD triple-mutant pituitaries. It is well known that bHLH factors, including Mash1, Math3 and NeuroD, form a heterodimer with a ubiquitously expressed bHLH factor, E47 and activate gene expression by binding to the E-box (Kageyama et al. 2005). It has been reported that E-box motifs are found in the promoters of FSHB and LHB (Kowase et al. 2007, Ciccone et al. 2008). Taken together, it is likely that Mash1, Math3 and NeuroD act independently of SF-1 and that they may directly control differentiation of FSH- and LH-secreting cells during pituitary development.

**Overexpression of Mash1 alone cannot induce pituitary endocrine cells**

It has been reported that a combination of three transcription factors, Mash1, Brn2 and Myt1 (BAM factors), are sufficient to convert mouse fibroblasts into functional neurons (Vierbuchen et al. 2010). Mash1 is also a central component of direct reprogramming of mouse fibroblasts to functional neurons (Wapinski et al. 2013). In the mouse cochlea, overexpression of Mash1 in non-sensory epithelial cells can induce neurons at embryonic, postnatal and juvenile stages (Nishimura et al. 2014). Mash1 alone can reprogram cochlear non-sensory epithelial cells into functional neurons. However, in mouse pituitary development, Mash1 alone could not induce pituitary endocrine cells, though it may be involved in differentiation of pituitary stem cells. To induce pituitary hormonal cells, a combination of Mash1 and other factors may be necessary.

**Conclusion**

Mash1, Math3 and NeuroD cooperatively control the timing of progenitor cell differentiation during adenohypophyseal development. Mash1, Math3 and NeuroD are also required for subtype specification of pituitary hormonal cells as follows. Mash1 is required for corticotroph and gonadotroph differentiation and compensated by Math3 and NeuroD. Math3 is necessary for somatotroph differentiation and compensated by Mash1 and NeuroD. Neurog2 may compensate for loss of Mash1, Math3 and NeuroD during pituitary development. In addition, Mash1, Math3 and NeuroD are required for pituitary development and compensate for each other.
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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