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Minireview

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The bHLH Gene *Hes1* Regulates Differentiation of Multiple Cell Types

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For embryos that have small pancreas and lack brain, eyes and thymus, the defects are caused by mutation of a single gene, *Hes1*. *Hes1* encodes a basic helix-loop-helix (bHLH) transcriptional repressor and functionally antagonizes positive bHLH genes such as the neuronal determination gene, *Mash1*. Mis-expression of *Hes1* inhibits cell differentiation and keeps cells at the precursor stage or proliferative stage. Conversely, in the absence of *Hes1*, the expression of positive bHLH genes is upregulated and cells differentiate prematurely without sufficient cell growth. As a result, the development of many tissues such as the brain, eye and pancreas is severely affected. Thus, *Hes1* regulates tissue morphogenesis by maintaining undifferentiated cells. In the case of T cell development, *Hes1* mutation leads to defects of expansion of early T cell precursors and thereby suppresses T cell fate specification. Thus, *Hes1* promotes differentiation of some cell types in addition to maintenance of the undifferentiated state. Interestingly, *Hes1* expression is controlled by the transmembrane protein Notch, which is activated by the ligands expressed on the surface of neighboring cells. Taken together, these results indicate that the Notch-Hes1 pathway, which is controlled by cell-cell interaction, plays an essential role in differentiation of many cell types.

Introduction

During embryogenesis, precursor cells initially proliferate to give rise to enough cells and, subsequently, these cells stop proliferation and start differentiation. The proper timing of transition from proliferation to differentiation is critical for normal development since premature or delayed transition leads to the abnormal cell numbers as well as abnormal morphology of the tissues. Recent studies revealed that these developmental processes are regulated positively or negatively by multiple basic helix-loop-helix (bHLH) genes in mammals as in *Drosophila* (Lee, 1997; Weintraub *et al*., 1991). For example, the bHLH genes *MyoD* and *Mash1* positively regulate muscle and neuronal determination/differentiation, respectively (Davis *et al*., 1987; Guillemot *et al*., 1993; Johnson *et al*., 1990), while the bHLH gene *Hes1* functionally antagonizes *MyoD* and *Mash1* and keeps cells at the precursor stage (Ishibashi *et al*., 1994; Sasai *et al*., 1992; Tomita *et al*., 1996). MyoD and Mash1 form a dimer with a ubiquitously-expressed bHLH factor such as E47, and each dimer activates specific gene expression by binding to the E box (CANNTG) whereas Hes1 inhibits the dimer formation of positive bHLH factors or represses the positive bHLH gene expression and thereby negatively regulates differentiation. The balance between the positive and negative bHLH genes is critical for the proper timing of differentiation and normal morphogenesis of various tissues (Kageyama and Nakanishi, 1997).

Among the multiple bHLH genes, *Hes1* may be one of the most important regulators for differentiation of various cell types: *Hes1* is expressed by almost all undifferentiated cells and is essential for the maintenance of these cells. Misexpression of *Hes1* inhibits differentiation of many cell types while *Hes1*-deficient mice show premature differentiation and severe defects in various tissues such as the brain, eye, and pancreas (Ishibashi *et al*., 1995; Jensen *et al*., 2000; Tomita *et al*., 1996). In addition, *Hes1* is also important for the generation of multiple cell types from common precursors, partly by maintaining the precursor pool at the time when different differentiation cues are present or by rather promoting differentiation of different cell types. Interestingly, *Hes1* expression is controlled by a transmembrane protein Notch (Jarriault *et al*., 1995; Nishimura *et al*., 1998), which is activated by ligands expressed by neighboring cells, and therefore cell-cell interaction is involved in regulation of *Hes1* expression. In

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this review, we will describe the function and regulation of *Hes1* in differentiation of various cell types.

Hes1 is a transcriptional repressor

Hes1 was originally identified as a mammalian homologue of *Drosophila hairy* and *Enhancer of split* (Sasai *et al*., 1992), which are known to inhibit neurogenesis in *Drosophila*. There are at least five members in mouse *Hes* family and each encodes a bHLH factor (Fig. 1). Each Hes factor forms a homodimer and a heterodimer through the helix-loop-helix region although it remains to be determined whether Hes homodimers and heterodimers have any different activities. In the basic region, a proline residue is uniquely conserved in the Hes family (Fig. 1, asterisk). Unlike most other bHLH factors, which bind to the E box (CANNTG), Hes1 binds to the N box (CACNAG) with a high affinity and to the E box only with a low affinity (Sasai *et al*., 1992). This difference of the DNA-binding specificity between Hes and other bHLH factors could be due to the uniquely conserved proline in the basic region, which is not conserved in E box-binding bHLH factors. Another structural feature is the carboxy-terminal four amino acid sequence Trp-Arg-Pro-Trp (the WRPW domain). It has been shown that the corepressor protein Groucho (or its mammalian homologues TLE/Grg) interacts with this sequence (Fisher *et al*., 1996; Paroush *et al*., 1994). Thus, all the Hes factors form a complex with Groucho and can repress transcription by directly binding to the N box (Akazawa *et al*., 1992; Sasai *et al*., 1992) (Fig. 2A). This Groucho-mediated process is called "active repression" and therefore Hes1 is categorized as an active repressor.

Hes1 can also repress transcription by a different mechanism. Hes1 can form a heterodimer with other bHLH factors such as E47 but this dimer cannot bind to the E box (Sasai *et al*., 1992). Thus, Hes1 represses transcription by sequestering positive bHLH factors (Fig. 2B). This mechanism is called "passive repression". Therefore, Hes1 functions as an active and passive repressor.

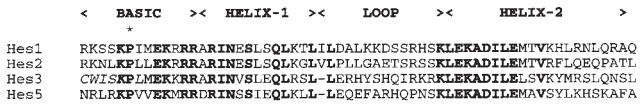

Fig. 1. The bHLH domain of Hes factors. The bHLH domain of Hes1, Hes2, Hes3 and Hes5 (Akazawa *et al*., 1992; Ishibashi *et al*., 1993; Sasai *et al*., 1992). The conserved amino acid residues are shown in bold. The conserved proline residue in the basic region is indicated by an asterisk.

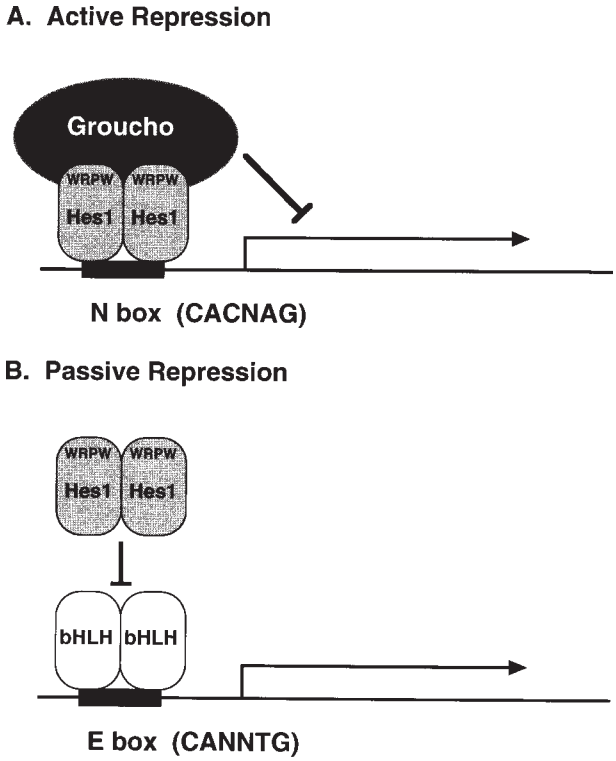

Fig. 2. Transcriptional repression by Hes1 (adopted from Kageyama and Ohtsuka, 1999). **A.** Active repression. Hes1 forms a dimer and binds to the N box. The corepressor Groucho interacts with the carboxy-terminal WRPW and mediates active repression. **B.** Passive repression. Most bHLH factors bind to the E box and activate gene expression. Hes1 shows a dominant-negative effect on these bHLH activators by sequestration.

Roles of *Hes1* in neural development

In the developing nervous system, neural precursor cells are present in the innermost layer called the ventricular zone while differentiating or mature neurons migrate out of the ventricular zone into the outer layers. *Hes1* is specifically expressed by neural precursor cells and the expression disappears when cells start differentiation (Sasai *et al*., 1992). Misexpression of *Hes1* with retrovirus inhibits neuronal and glial differentiation, and the cells expressing *Hes1* most likely remain as precursor cells (Castella *et al*., 1999; Ishibashi *et al*., 1994; Tomita *et al*., 1996). This suppressive effect of *Hes1* on differentiation is partly due to inhibition of the positive bHLH factors such as Mash1 (Sasai *et al*., 1992).

Conversely, in *Hes1*-deficient mice, maintenance of neural precursor cells is impaired and these cells prematurely differentiate into neurons. As a result, the neural tube formation process is disturbed particularly in the cranial region, resulting in exencephaly and anencephaly (Ishibashi *et al*., 1995) (Fig. 3). In addition, optic vesicle formation is also severely disturbed by

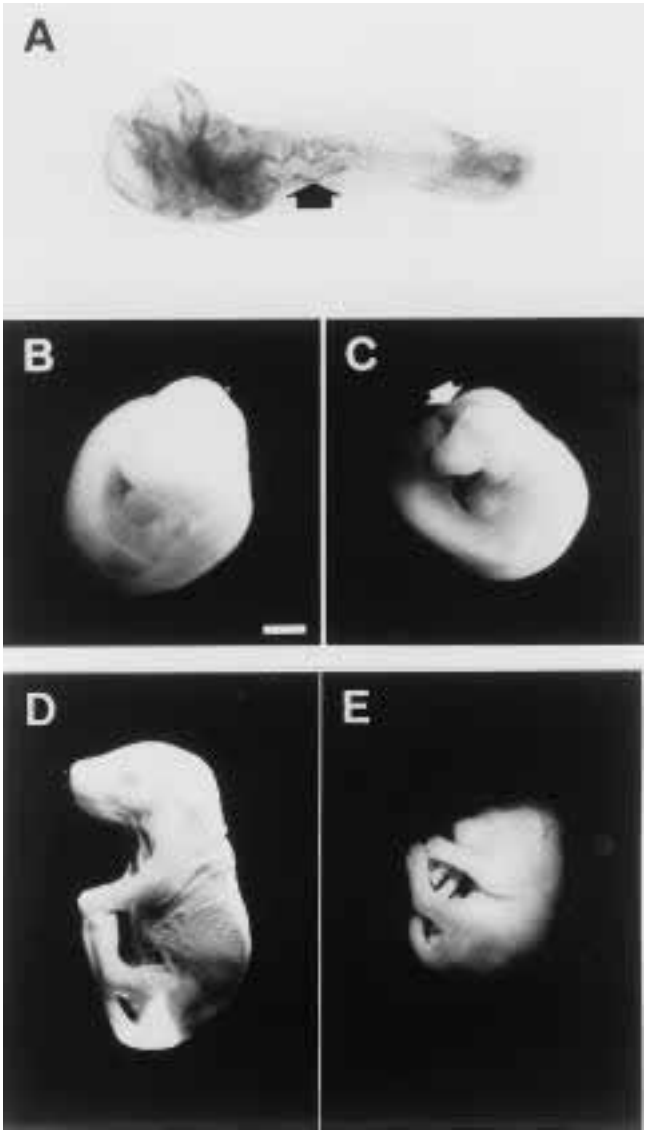

Fig. 3. Neural tube defects of *Hes1*-deficient embryos (adopted from Ishibashi *et al*., 1995). Wild-type (**B**, **D**) and *Hes1*-deficient embryos (**A**, **C**, **E**). *Hes1*-deficient embryos show a kinked neural tube (**A**, arrow), the open brain (**C**, arrow) and anencephaly (**E**). These neural tube defects are associated with reduced growth of neural precursor cells and premature neuronal differentiation.

premature differentiation and *Hes1*-deficient mice have small eyes in most cases or no eyes at all in some extreme cases (Tomita *et al*., 1996). Furthermore, whereas the number of early-differentiating neurons seems normal or is sometimes increased in the absence of *Hes1*, that of late-differentiating neurons is severely reduced (Nakamura *et al*., 2000; Tomita *et al*., 1996). This is probably the result of the lack of neural precursor cells at the time of differentiation of late neurons because of premature differentiation. Thus, *Hes1* is very important for the correct timing of differentiation and for generation of the correct number of cells. In *Hes1*-deficient mice, the expression of the positive bHLH genes such as *Mash1* is upregulated, and therefore it is likely that upregulation of *Mash1* expression may account for premature neuronal differentiation. It was shown that Hes1 directly binds to the Mash1 promoter and represses transcription of the *Mash1* gene (Chen *et al*., 1997). Since Hes1 can also suppress Mash1 activity by sequestration, these results indicate that Hes1 inhibits Mash1 at both transcriptional and post-transcriptional levels.

Roles of *Hes1* in muscle development

The bHLH factor MyoD forms a heterodimer with E47, binds to the E box in the muscle-specific promoter and determines the myogenic fate (Weintraub *et al*., 1991). In contrast, Hes1 inhibits DNA binding of MyoD-E47 heterodimer by sequestration and thereby inhibits MyoD-induced myogenesis (Sasai *et al*., 1992). However, *Hes1*-deficient mice show no apparent muscle defects (Ishibashi *et al*., 1995). Therefore, it remains to be determined whether Hes1 really regulates the timing of muscle differentiation like neurogenesis.

Roles of *Hes1* in exocrine and endocrine development

Endocrine and exocrine cells differentiate from common precursor cells. Recent studies revealed that endocrine cell development is positively regulated by the same set of bHLH genes as used for neurogenesis, such as *Neurogenin*, *NeuroD* and *Mash1*, that promote neuronal differentiation (Apelqvist *et al*., 1999; Borges *et al*., 1997; Naya *et al*., 1997). Overexpression of *Neurogenin3* accelerates endocrine cell differentiation and blocks exocrine cell development (Apelqvist *et al*., 1999). In contrast, activation of the Notch pathway increases *Hes1* expression and keeps cells at the precursor stage or adopts the exocrine cell fate probably by antagonizing the positive bHLH genes (Apelqvist *et al*., 1999). Conversely, in *Hes1*-deficient mice, expression of positive bHLH genes such as *Neurogenin3* is upregulated and endocrine cells differentiate prematurely (Jensen *et al*., 2000). As a result, precursor cell maintenance and exocrine cell development are disturbed and therefore *Hes1*-deficient mice have severe defects of pancreas and gut morphogenesis (Jensen *et al*., 2000). These results indicate that, as in neurogenesis, the balance between the positive bHLH genes and *Hes1* is critical for exocrine and endocrine development.

Roles of *Hes1* in T cell development

Hes1 is expressed in the developing T cells in the thymus. Surprisingly, *Hes1*-deficient mice have no thymus or small thymus (Figs. 4B and 4D) while other hematopoietic lineages seem to be unaffected (Tomita *et al*., 1999). Even

when a small thymus is present in some *Hes1*-deficient embryos, mature T cells are not detected (Fig. 4F). Since *Hes1* is expressed by both thymocytes and thymic stromal cells, the thymic phenotypes of *Hes1* mutation *per se* do not tell which cell types are responsible for the thymic defects. However, reconstitution of the lymphoid lineages of *RAG2*-deficient mice with *Hes1*-null fetal liver cells shows that, whereas B cells develop normally, mature T cells are not generated in the thymus (Tomita *et al*., 1999). Therefore, *Hes1* is specifically required for T cell lineage, and T cell development is cell-autonomously affected by *Hes1* mutation. In the absence of *Hes1*, T cell development is arrested at the $CD4^{-}CD8^{-}$ (DN) stage. The DN stage is further divided into four distinct stages by the surface markers CD44 and CD25 (Godfrey and Zlotnik, 1993). At the first stage ($CD44^{+}CD25^{-}$), DN cells are resting but start proliferating before T cell receptor (TCR) gene rearrangement occurs at the next stage ($CD44^{+}CD25^{+}$). During the third stage ($CD44^{-}CD25^{+}$), DN cells undergo rearrangement of TCRβ locus and, only when the β locus is productively rearranged do not DN cells selectively proliferate and differentiate into $CD4^{+}CD8^{+}$ DP cells after the $CD44^{-}CD25^{-}$ DN stage. In the absence of *Hes1*, T cell development is arrested at the earliest stage ($CD44^{+}CD25^{-}$) before TCR gene rearrangement occurs (Tomita *et al*., 1999). Since T cell fate is not determined before the TCR gene rearrangement stage, the defects observed in *Hes1* mutation indicate that *Hes1* plays an important role in T cell fate specification at the earliest stage. Even though a very minor population of *Hes1*-null thymocytes could undergo TCR gene rearrangement, these cells still do not expand. Thus, *Hes1* is essential for expansion of earliest T cell precursors. Remarkably, this defect is very similar to the defect of *Notch1*-null mutation (Radtke *et al*., 1999), raising the possibility that Notch and Hes1 function in the same pathway for specification of the T cell fate (Osborne and Miele, 1999).

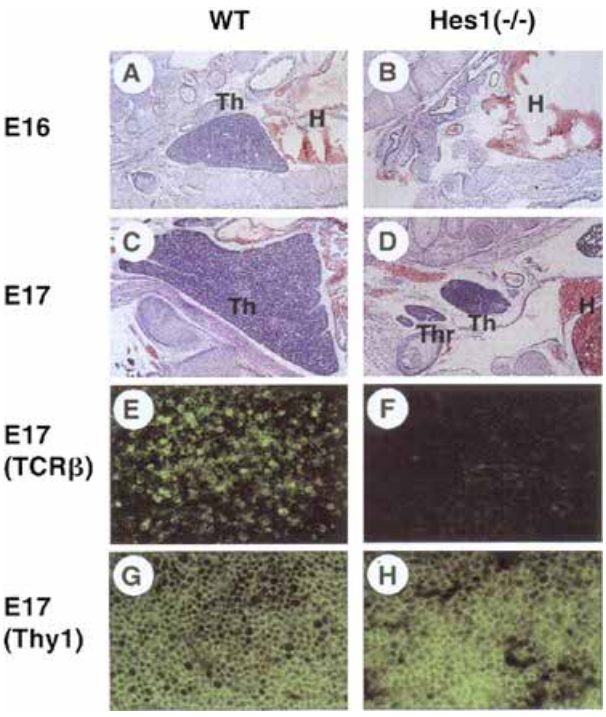

Fig. 4. Defects of thymus development of *Hes1*-deficient mice (adopted from Tomita *et al*., 1999). Sections of wild-type (**A**, **C**, **E**, **G**) and *Hes1*-deficient embryos (**B**, **D**, **F**, **H**). *Hes1*-deficient embryos have no thymus (**B**) or small thymus (**D**). In *Hes1*-deficient thymus, there are $Thy1^{+}$ thymocytes (**H**) but no mature T cells (**F**). Thus, *Hes1* is essential for T cell development. H, heart; Th, thymus; Thr, thyroid.

Hes1 is also involved in the later stage of T cell development. DP cells differentiate into $CD4^{+}$ SP cells or $CD8^{+}$ SP cells. It has been shown that during $CD8^{+}$ SP cell development CD4 gene expression is specifically repressed. This repression involves the presence of a silencer region in the intron of the *CD4* gene (Sawada *et al*., 1994; Siu *et al*., 1994). Interestingly, the silencer region contains the N box, and misexpression of *Hes1* in DP cells reduces *CD4* expression (Kim and Siu, 1998). This effect is again very similar to the effect by activation of *Notch1* (Robey *et al*., 1996), suggesting that activation of the Notch-Hes1 pathway may lead to repression of CD4 expression and differentiation of $CD8^{+}$ SP cells. Thus, *Hes1* is involved in at least two different stages of T cell development.

The Notch-Hes1 pathway

Since *Hes1* is essential for normal development, its expression should be carefully controlled. Recent studies revealed that *Hes1* expression is transcriptionally controlled by the transmembrane protein Notch (Jarriault *et al*., 1995; Nishimura *et al*., 1998), which is known to regulate maintenance of precursor cells and generation of cell type diversity. Notch is activated by its ligands such as Delta and Jagged, which are expressed by the surface of neighboring cells (Artavanis-Tsakonas *et al*., 1999). Thus, cell-cell interaction influences the activity of Notch signaling. Upon activation, the intracellular domain of Notch is cleaved and released from the membrane region. It is suggested that this cleavage is regulated by *Presenilin*, a gene responsible for Alzheimer's disease (Chan and Jan, 1999). After cleavage, the intracellular domain of Notch (ICD) is translocated into the nucleus since it has a nuclear localization signal. In the nucleus, ICD forms a complex with the DNA-binding protein RBP-J (Honjo, 1996; Kageyama and Ohtsuka, 1999). RBP-J is a transcriptional repressor, but when it forms a complex with ICD, the complex becomes an transcriptional activator. This complex binds to the *Hes1* promoter and induces *Hes1* expression. In addition to *Hes1*, expression of another *Hes*

member, *Hes5*, is also regulated by Notch (de la Pompa *et al*., 1997; Nishimura *et al*., 1998).

The functional linkage between Notch and *Hes1*/*Hes5* is shown by using *Hes1* and *Hes5* mutant mice (Ohtsuka *et al*., 1999). Misexpression of ICD in neural precursor cells inhibits neuronal differentiation. Likewise, ICD inhibits differentiation of neural precursor cells prepared from *Hes1*-deficient and *Hes5*-deficient embryos (Fig. 5), suggesting that either *Hes1* or *Hes5* is sufficient for the Notch activity. In contrast, ICD fails to inhibit differentiation of neural precursor cells prepared from *Hes1*- and *Hes5*-double-deficient embryos (Fig. 5), suggesting that *Hes1* and *Hes5* are essential effectors in the Notch pathway. However, differentiation of some neurons is still inhibited by ICD even in the absence of both *Hes1* and *Hes5* (Ohtsuka *et al*., 1999), raising the possibility that there may be other molecules that mediate the Notch function.

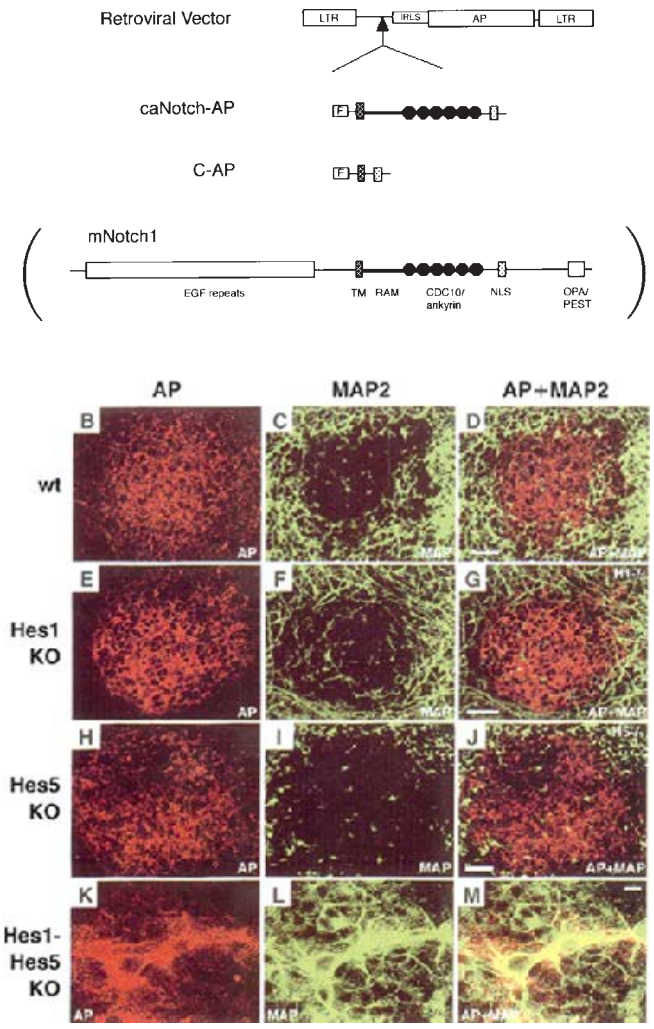

Fig. 5. *Hes1* and *Hes5* as essential Notch effectors (adopted from Ohtsuka *et al*., 1999). **A.** Schematic structure of the recombinant retroviruses. caNotch-AP virus directs expression of a constitutively active form of Notch, which consists of the RAM, ankyrin repeats, and nuclear localization signal. Alkaline phosphatase (AP) is also expressed through the IRES (internal ribosomal entry site). (**B–M**) Neural precursor cells were prepared from wild type (**B–D**), *Hes1*-null (**E–G**), *Hes5*-null (**H–J**), and *Hes1-Hes5* double-null mouse embryos (**K–M**). These cells were infected with caNotch-AP and, two weeks after infection, the fate of the virus-infected cells (AP^+) was determined. caNotch-AP infection inhibited neuronal differentiation of wild-type, *Hes1*-null, and *Hes5*-null cells (AP^+, $MAP2^-$) but not of *Hes1-Hes5* double-null cells (AP^+, $MAP2^+$). Thus, *Hes1* and *Hes5* are essential for Notch-induced inhibition of neuronal differentiation.

Post-translational regulation of Hes1 during differentiation

For transition to differentiation, downregulation of Hes1 activity is essential. However, the precise mechanism of inactivation of Hes1 during development is not well understood. Inactivation of Notch is obviously one of the main mechanisms, but it is demonstrated that post-translational modification is also very important. There are two adjacent serine residues in the basic region of Hes1 and these residues are phosphorylated by kinases such as PKC. A phosphorylated form of Hes1 loses the DNA-binding activity and therefore is unable to inhibit differentiation (Ström *et al*., 1997). Phosphorylation of Hes1 is observed during NGF-induced neuronal differentiation, and misexpression of a non-phosphorylated mutant form of Hes1 in which the two serine residues are changed to valine and methionine inhibits NGF-induced neuronal differentiation (Ström *et al*., 1997). Thus, post-translational modification of Hes1 is essential for transition to differentiation.

Conclusion

In this review, we described the functions of *Hes1* in the differentiation process of various cell types (Fig. 6). In neural, myogenic and exocrine/endocrine development, *Hes1* keeps cells at the precursor stage and induces cell proliferation. In exocrine/endocrine development, *Hes1* is also involved in exocrine cell fate specification. Thus, *Hes1* seems to have two functions: maintaining undifferentiated cells and promoting alternative cell fate during binary cell fate choice. The function of *Hes1* in the selection of one cell fate from the other is typically observed during T cell versus B cell fate determination (Fig. 6). Activation of the Notch-Hes1 pathway specifies the T cell fate while inactivation of the pathway specifies the B cell fate (Pui *et al*., 1999; Radtke *et al*., 1999; Tomita *et al*., 1999). Thus, *Hes1* seems to have different functions depending on cell types. However, these functions could be actually the same: *Hes1* chooses the undifferentiation from the undifferentiation/differentiation choice and selects the T cell and exocrine cell fate from the other cell types.

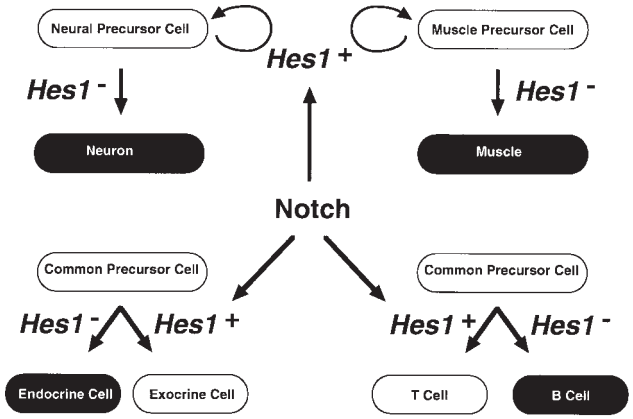

Fig. 6. Notch-Hes1 pathway in differentiation of multiple cell types. In neural development *Hes1* directs maintenance of neural precursor cells, and in muscle development *Hes1* directs maintenance of muscle precursor cells. In contrast, in endocrine/exocrine development *Hes1* directs exocrine development, and in lymphopoiesis *Hes1* directs T cell development. In all cases, *Hes1* makes one choice from the other.

Thus, the main and common function of *Hes1* may be making one choice from the other. This *Hes1* function involves the inhibition of positive bHLH genes such as *Mash1*. However, the precise mechanism of how *Hes1* keeps a proliferative state is not yet known, and further study is necessary to further understand *Hes1* functions.

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