The cyclic gene *Hes1* contributes to diverse differentiation responses of embryonic stem cells

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Stem cells do not all respond the same way, but the mechanisms underlying this heterogeneity are not well understood. Here, we found that expression of *Hes1* and its downstream genes oscillate in mouse embryonic stem (ES) cells. Those expressing low and high levels of Hes1 tended to differentiate into neural and mesodermal cells, respectively. Furthermore, inactivation of *Hes1* facilitated neural differentiation more uniformly at earlier time. Thus, *Hes1*-null ES cells display less heterogeneity in both the differentiation timing and fate choice, suggesting that the cyclic gene *Hes1* contributes to heterogeneous responses of ES cells even under the same environmental conditions.

Supplemental material is available at http://www.genesdev.org.

Embryonic stem (ES) cells have the ability to differentiate into multiple cell types of all three germ layers, providing powerful tools to realize mammalian early development in culture dishes as well as to create specific types of cells for regenerative medicine (Smith 2001; Murry and Keller 2008). Many methods for triggering in vitro differentiation of ES cells have been established, but most of them require additional purification to collect homogeneous cell populations. One main reason for such heterogeneity is that ES cells asynchronously differentiate into diverse cell types, and thereby give rise to mixtures of differentiated and undifferentiated cells in a rather chaotic manner (Lowell et al. 2006). Recent studies have demon-

strated that expression of the homeodomain factor Nanog and the zinc finger protein Rex1 fluctuates over several days in individual ES cells (Chambers et al. 2007; Singh et al. 2007; Toyooka et al. 2008). The phenotypic output of this heterogeneity appears during their differentiation: Nanog-negative ES cells are prone to differentiate into primitive endoderm-like cells (Chambers et al. 2007; Singh et al. 2007) while Rex1-negative ES cells correspond to epiblast and primitive ectoderm and more rapidly differentiate into somatic lineages (Toyooka et al. 2008). These studies indicate that ES cells are not homogeneous. Rather, they are heterogeneous in differentiation competency (Chambers et al. 2007; Toyooka et al. 2008). However, even Nanog-positive ES cells are not homogeneous, and the molecular mechanism underlying this heterogeneity is not well understood, although such heterogeneity is suggested to be important for dynamic differentiation process of ES cells, based on theoretical analysis of cell systems (Furusawa and Kaneko 2001; Kaneko 2006).

Hes1 is a member of the basic helix–loop–helix (bHLH) transcriptional repressors that regulate cell proliferation and differentiation in embryogenesis (Kageyama et al. 2007). We previously found that Hes1 is expressed in an oscillatory manner with a period of ~2 h in various cells such as fibroblasts and neural progenitor cells (Hirata et al. 2002; Shimojo et al. 2008). This oscillatory expression is regulated by negative feedback and rapid degradation of the gene products: Hes1 can repress its own expression, but rapid degradation of both Hes1 mRNA and protein with half-lives of ~20 min soon results in the release from negative regulation and allows the next round of expression (Hirata et al. 2002). We here found that Hes1 expression also oscillates in ES cells and that *Hes1*-null ES cells display less heterogeneity in both the differentiation timing and fate choice, suggesting that the cyclic gene *Hes1* contributes to heterogeneous responses of ES cells even under the same environmental conditions.

Results and Discussion

Hes1 expression oscillates in ES cells

Hes1 protein was highly expressed by ES cells under the control of two essential factors, LIF and BMP (Fig. 1A, lanes 2–6; Ying et al. 2003), but not of Notch signaling (Fig. 1A, lane 7). Interestingly, Hes1 expression levels were variable in individual ES cells while Oct3/4 also seemed to be variable to some extent (Fig. 1B). To determine whether Hes1 expression oscillates in ES cells, we employed a real-time imaging method using a *Hes1* promoter-driven ubiquitin-fused firefly luciferase reporter (Fig. 1C) that was shown previously to mimic the endogenous *Hes1* expression (Masamizu et al. 2006). Indeed, the *Hes1* promoter-driven reporter expression oscillated in individual ES cells (Fig. 1C,D; Supplemental Movie), whereas the human polypeptide chain elongation factor 1α (EF-1α) promoter-driven reporter expression did not (data not shown). These results indicated that *Hes1* expression oscillates in ES cells.

The oscillations continued throughout the cell cycle, but the Hes1 tended to be expressed at higher levels during S–G2 phases compared with G1 phase (data not shown). The oscillations seemed to be synchronized between neighboring daughter cells after cell division,
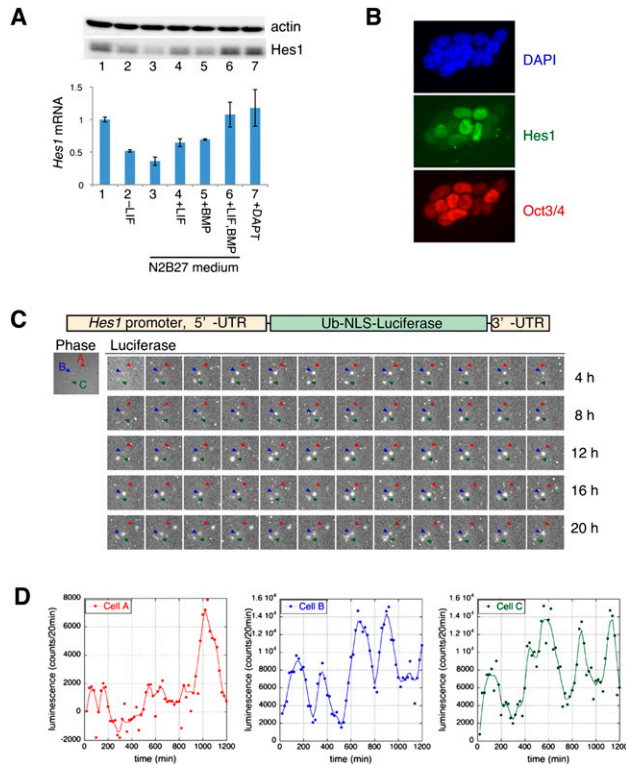
Figure 1. Oscillatory expression of Hes1 in mouse ES cells. (*A*) Hes1 protein (*top* panel) and mRNA (*bottom* panel) expression in normal (lane *1*) or other culture conditions, after the removal of LIF in normal ES medium (lane *2*), in N2B27 serum-free medium (lane *3*), in the presence of LIF (lane *4*), BMP (lane *5*), or both LIF and BMP (lane *6*) in N2B27 medium, and in the presence of DAPT, a γ-secretase inhibitor (Notch signaling inhibitor), in normal ES medium (lane *7*). *Hes1* mRNA levels were analyzed by Q-PCR, normalized by GAPDH and given in ratios to ES cells cultured in normal ES medium. (*B*) Immunocytochemistry using anti-Hes1 (green) and anti-Oct4 antibodies (red) with DAPI (blue). (*C*) Bioluminescence images were taken by 20-min exposures for 20 h from three ES cells that express the ubiquitin (Ub)-fused and nuclear localization signal (NLS)-fused firefly luciferase reporter under the control of the *Hes1* promoter (see the Supplemental Movie). (*D*) Quantification of the bioluminescence activity of cells A (red arrowhead), B (blue arrowhead), and C (green arrowhead) shown in *C*.

although they easily became out of synchrony in large colonies (data not shown). The period of *Hes1* oscillations was variable from cycle to cycle and from cell to cell (Fig. 1D). The power spectrum of *Hes1* oscillations after Fourier transform showed that the periodicity was ∼3–5 h, although it included many noises of short periodicity (Supplemental Fig. S1). The period of 3–5 h in ES cells was longer than in other cell types (2–3 h) (Hirata et al. 2002; Masamizu et al. 2006; Shimojo et al. 2008). The half-life of Hes1 protein was ∼16 min in ES cells, which was similar to fibroblasts, whereas that of *Hes1* mRNA was about two to four times longer in ES cells (∼46 min) than in fibroblasts (data not shown), implying that the stabilization of *Hes1* mRNA contributes to a longer periodicity in ES cells.

Hes1 represses expression of Notch ligand and cell cycle regulator genes

To elucidate the role of Hes1 in ES cells, we next sought to determine the downstream target genes. To this end,

we established two types of ES cells (two or three independent lines for each type), *Hes1*-null and *Hes1*-sustained cells. *Hes1*-null (K3, K9, and K57) cells were made by transient Cre expression in ES cells carrying *Hes1*-floxed and *Hes1*-null alleles (Fig. 2A, lanes 2,3; Supplemental Fig. S2). *Hes1*-sustained (R5 and R6) cells were made by knocking *Hes1* cDNA into the Rosa26 locus (Supplemental Fig. S3), and these cells expressed Hes1 in a sustained manner, whose levels were similar to the endogenous maximal level (Fig. 2A, lanes 5,6; Supplemental Fig. S4). Both *Hes1*-null and *Hes1*-sustained cells expressed similar levels of *Oct3/4, Sox2, Nanog,* and *Rex1,* markers of ES cells (Fig. 2B), and proliferated on feeder cells at similar levels to wild-type ES cells (data not shown). Furthermore, both types of ES cells were able to form three germ layers in embryoid body (Supplemental Fig. S5) and chimeric embryo formation assays (Supplemental Fig. S6), indicating that the proliferative and
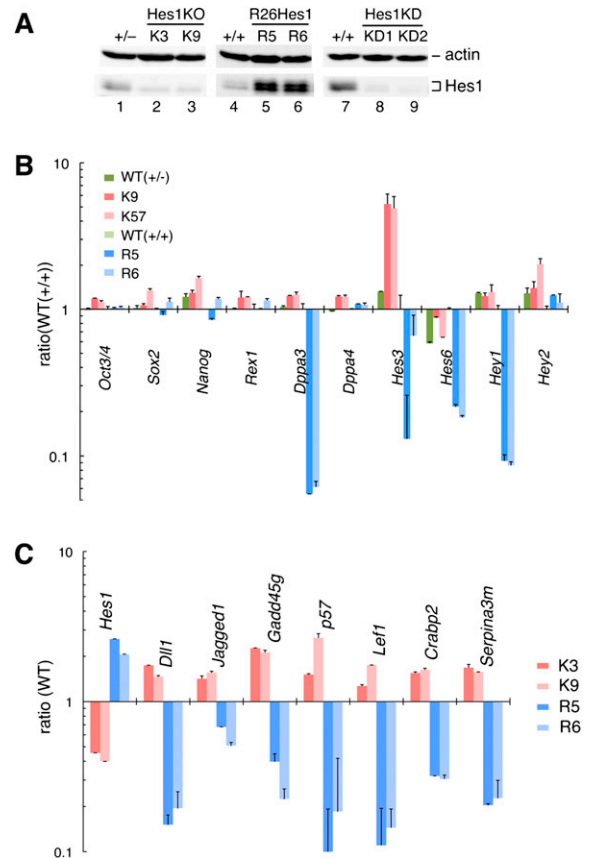

Figure 2. Comparison of gene expression between *Hes1*-null and *Hes1*-sustained ES cells. (*A*) Hes1 protein levels in *Hes1*-null (K3 and K9; lanes *2,3*), *Hes1*-sustained (R5 and R6; lanes *5,6*), *Hes1* knockdown (KD1 and KD2; lanes *8,9*), and parental cells (+/−, +/+; lanes *1,4,7*). *Hes1* knockdown cells were made by two different shRNA constructs (KD1, KD2). Actin is a loading control for Western blotting. (*B*) The mRNA levels of ES cell marker genes (*Oct3/4, Sox2, Nanog, Rex1, Dppa3,* and *Dppa4*) and Hes family genes (*Hes3, Hes6, Hey1,* and *Hey2*). They were analyzed by Q-PCR and given in ratios to wild-type (+/+) ES cell line (lane *4* in *A*). Most of ES cell markers, except *Dppa3,* were expressed at similar levels in all cell lines. *Hes5* expression was very low in all ES cells (data not shown). (*C*) The mRNA levels of Hes1 target genes in ES cell lines (+/−, +/+, K3, K9, R5, and R6) were analyzed by Q-PCR and given in ratios to their parental wild-type cells.

multipotential activities are maintained in both *Hes1*-null and *Hes1*-sustained ES cells. We searched the downstream genes of Hes1 by comparing mRNA expression levels between wild-type, R5/R6 and K3/K9 cells using a microarray method. We found that 36 genes displayed more than twofold higher expression in K3/K9 (*Hes1*-null) cells than in R5/R6 (*Hes1*-sustained) cells on microarray analysis, suggesting that these genes are repressed by *Hes1* (Supplemental Table S1). Among them, seven genes were confirmed as *Hes1* targets by real-time PCR, including two Notch ligands, *Dll1* and *Jagged1* (*Jag1*), and two cell cycle inhibitory genes, *Gadd45g* (Vairapandi et al. 2002) and *p57* (Fig. 2C). We also performed chromatin immunoprecipitation (ChIP) by using Hes1 antibody and subjected the DNA precipitates to oligonucleotide tiling arrays (ChIP–chip) (Wendt et al. 2008) to identify direct target genes for Hes1. We used Hes1 knockdown ES cells as a negative control (Fig. 2A, lanes 8,9). Five-hundred-six Hes1-binding regions were identified, and the list contained many gene loci for transcription factors that regulate cell differentiation as well as Notch signaling, suggesting that Hes1 is involved in ES cell differentiation (Supplemental Table S2). Among the genes identified by the expression array, *Dll1*, *Jag1*, *Gadd45g*, *Lef1*, *Crabp2*, and *Hes1* were also identified by ChIP–chip analysis.

Expression of some downstream genes also oscillates in ES cells

Because *Hes1* expression oscillated, some downstream genes might be also expressed in an oscillatory manner. To determine the relationship of *Hes1* expression with the downstream gene expression, we randomly picked up 32 single ES cells and made cDNAs from each cell to perform quantitative real-time PCR (single-cell Q-PCR) (Kurimoto et al. 2006, 2007). These ES cells expressed variable levels of *Hes1* and were classified into three groups according to the *Hes1* expression level (Fig. 3A). When *Hes1* expression was high, both *Dll1* and *Gadd45g* expression was also high and vice versa, whereas the other downstream genes *p57*, *Lef1*, *Jag1*, and *Crabp2* displayed different patterns (Fig. 3B; data not shown). Thus, expression levels of *Hes1*, *Dll1*, and *Gadd45g* changed in a similar manner in individual ES cells, suggesting that these genes oscillate in phase. When the Hes1 protein level is high, transcription of all *Hes1*, *Dll1*, and *Gadd45g* genes may be repressed, but they may be activated when the Hes1 protein level is low. Due to this delayed negative regulation by Hes1, expression of all these genes probably oscillated in phase. To obtain direct evidence that both *Dll1* and *Gadd45g* expression also oscillate in ES cells, we further analyzed their expression dynamics by a real-time imaging method using a ubiquitin-fused luciferase reporter under the control of *Dll1* or *Gadd45g* promoter. The expression of both *Dll1* and *Gadd45g* dynamically changed in many individual ES cells (Fig. 3C). These results suggest that the differentiation competency of ES cells can be changed rapidly by oscillations of *Dll1*, a ligand of Notch signaling that induces neural differentiation (Lowell et al. 2006), and *Gadd45g*, which inhibits cell cycle progression (Vairapandi et al. 2002). Interestingly, while *Nanog* was mostly expressed by picked-up ES cells (Fig. 3A), its expression level seemed to be variable and became higher when *Hes1* was highly expressed (Fig. 3B), whereas there
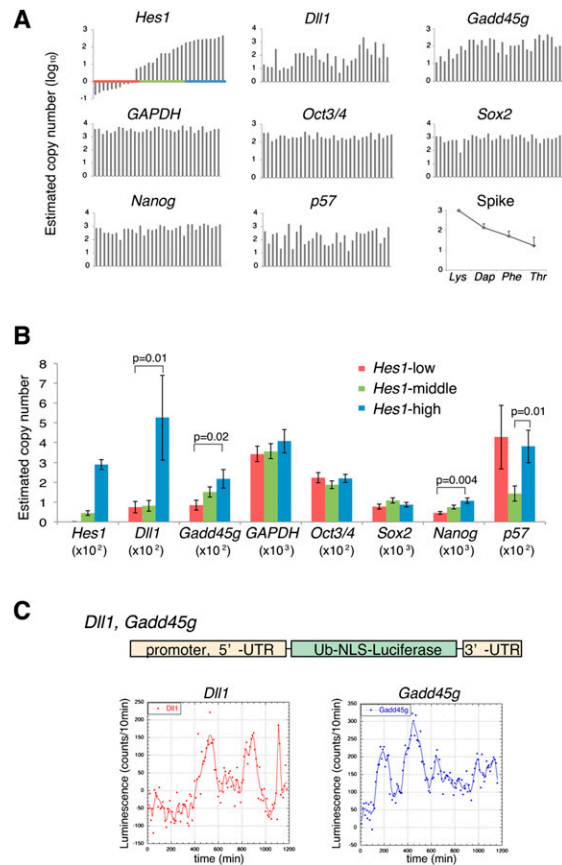

Figure 3. Dynamic expression of Hes1 target genes in ES cells. (*A*) Single-cell Q-PCR analysis with 32 randomly picked up ES cells. The results were aligned along the x-axis according to the *Hes1* expression levels. Graphs show the putative number (log10) of transcript of *Hes1*, Hes1 downstream genes (*Dll1*, *Gadd45g*, and *p57*), a housekeeping gene (*GAPDH*), and ES cell marker genes (*Oct3/4*, *Sox2*, and *Nanog*) in each single cell. (*Bottom right*) Control spike RNAs (*Lys*, *Dap*, *Phe*, and *Thr* for 1000, 100, 20, and 5 copies, restrictively) were quantified by Q-PCR and plotted by the average of all cDNA samples with standard errors. (*B*) Cells were classified into three groups according to the *Hes1* expression levels: *Hes1*-low (red), *Hes1*-middle (green) and *Hes1*-high (blue), shown in *A*. The average transcript number in each population was plotted with standard errors (normal scale). *P*-value was calculated by Mann-Whitney *U*-test. (*C*) Real-time imaging of *Dll1* and *Gadd45g* expression in ES cells was performed by using *Dll1* promoter-driven or *Gadd45g* promoter-driven ubiquitin-fused luciferase reporter. Bioluminescence images were taken by 10-min exposures for 20 h, and representative plots of luminescence in each single cell were shown.

was no such relationship with *Oct3/4* and *Sox2* (Fig. 3B), suggesting that *Hes1* oscillations may have some correlation with *Nanog* fluctuation (Chambers et al. 2007) but not with *Oct3/4* or *Sox2*.

Hes1-high and Hes1-low ES cells display different responses

We showed that *Hes1* expression oscillates in ES cells and that this *Hes1* oscillation leads to oscillatory expression of some of the downstream genes that regulate Notch signaling and the cell cycle. We next asked whether different *Hes1* expression levels contribute to heterogeneous responses in the differentiation of ES cells. We

reasoned that ES cells could respond differently to the differentiation signal according to the Hes1 expression levels at a given time. To address this issue, the Venus (a YFP variant) fragment was knocked into the Hes1 locus of ES cells so that the Venus-Hes1 fusion protein was expressed under the control of the endogenous Hes1 promoter. These cells were found to express variable levels of the Venus-Hes1 fusion protein, and the Venus fluorescence intensity well correlated with the endogenous Hes1 protein levels (Fig. 4A).

Using fluorescence-activated cell sorting (FACS), Hes1-high and Hes1-low ES cells were isolated and further characterized (Fig. 4B,C). These cells returned to the original distribution of variable expression levels within 1 d (Fig. 4D), indicating that Hes1-high and Hes1-low cells represent different phases of Hes1 oscillations rather than different subpopulations of ES cells. We next transferred Hes1-high and Hes1-low cells to the neural differentiation medium (Ying and Smith 2003) immediately after

Figure 4. Hes1-high and Hes1-low ES cells are prone to mesodermal and neural differentiation, respectively. (A) Venus-Hes1 knockin ES cells on feeder cell layer. Detailed information about these ES cells will be described elsewhere. Phase contrast view and merged images of immunofluorescence using anti-GFP (green) and anti-Hes1 antibodies (red) with DAPI (blue). Bar, 20 μm. (B) Venus-high cells (blue) and Venus-low cells (red) were sorted by flow cytometry. FSC, forward scatter. (C) Histogram of whole cells (green, *top* panel) and two fractions of Venus-high and Venus-low cells (blue and red, *bottom* panel). (D) Hes1-high and Hes1-low ES cells returned to the original distribution of variable expression levels within 1 d when they were cultured in ES medium. (E) mRNA levels of *Oct3/4* and *Nanog* were analyzed by Q-PCR of Hes1-high and Hes1-low ES cells just after sorting into the N2B27 medium. Each value was given in the ratio to unsorted cells. (F) Hes1-high and Hes1-low cells were sorted and directly transferred into the N2B27 differentiation medium and cultured for 4 d. Gene expression was quantified by Q-PCR. Each value was given in the ratio to unsorted cells (day 0).

sorting. Both sorted cells initially expressed *Oct3/4* and *Nanog* at comparable levels (Fig. 4E). Although there was no significant difference in *Oct3/4* decrease and *Fgf5* increase, a primitive-ectoderm marker (Kunath et al. 2007), associated with differentiation (Fig. 4F), Hes1-low cells expressed a higher level of the neural marker *Mash1* and the neuronal marker Tuj-1 than Hes1-high cells at day 4 (Fig. 4F), suggesting that Hes1-low cells more efficiently differentiated into neurons. In contrast, Hes1-high cells expressed a higher level of the mesodermal marker *Brachyury* than Hes1-low cells at day 4 (Fig. 4F), suggesting that Hes1-high cells more efficiently differentiated into mesodermal cells. These results indicated that different *Hes1* expression levels contribute to heterogeneous responses in the cell fate choice. Interestingly, Hes1 oscillations were arrested after induction of differentiation but resumed later in somatic stem/progenitor cells (data not shown).

Inactivation of Hes1 *leads to accelerated and more uniform differentiation into the neural fate*

To obtain further evidence that *Hes1* is involved in heterogeneous responses of ES cells, we compared the differentiation timings and cell fate choice of *Hes1*-null and wild-type ES cells. We measured the expression kinetics of marker genes during differentiation after removal of both LIF and feeder cells, a condition known to induce all three germ layers (Rathjen and Rathjen 2001). Expression of all differentiation marker genes was activated in wild-type ES cells within 10 d (+/+ and +/− in Supplemental Fig. S5B), but up-regulation of *Mash1* was more pronounced in *Hes1*-null (K9 and K57) cells than in wild-type cells (Supplemental Fig. S5B).

To further characterize the role of *Hes1* in ES cells, we focused on neural differentiation because inactivation of *Hes1* promotes neuroectoderm differentiation (Supplemental Fig. S5B). Under a neural differentiation condition, *Hes1*-null ES cells (K9 and K57) expressed higher levels of *Mash1*, *Nestin*, *Tuj-1* (Fig. 5B), and Notch signaling molecules *Jag1*, *Dll1*, and *Hes5* (Fig. 5C), which are involved in neural induction (Lowell et al. 2006; Nemir et al. 2006), while they expressed lower levels of other marker genes—*Fgf5*, *Brachyury*, and *Gata4*—than wild-type cells (Fig. 5A). This preference to the neural fate agreed well with that of Hes1-low cells (Fig. 4F). Importantly, while wild-type ES cells only randomly differentiated into neural cells even under the neural differentiation condition, *Hes1*-null ES cells (K9) more uniformly differentiated into neural cells at earlier timings (Fig. 5D,E). At day 6, almost all *Hes1*-null ES cells differentiated into neural cells (either Nestin[+] neural progenitor cells or Tuj-1^{+} neurons), whereas only subsets of wild-type ES cells did so (Fig. 5D,E). Thus, inactivation of *Hes1* reduces heterogeneous responses and leads to more uniform and preferential differentiation of ES cells into the neural fate.

Recent reports showed that reversible changes in gene expression occur slowly, over several days, in ES cells and hematopoietic progenitor cells, resulting in different potentials for differentiation, although the mechanism for such slow changes remains to be elucidated (Chambers et al. 2007; Chang et al. 2008; Graf and Stadtfeld 2008; Hayashi et al. 2008; Toyooka et al. 2008). We here showed
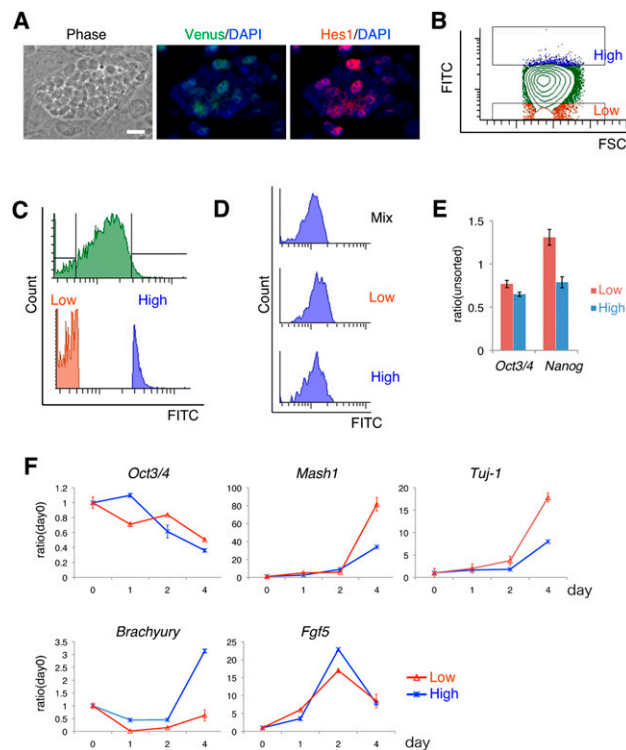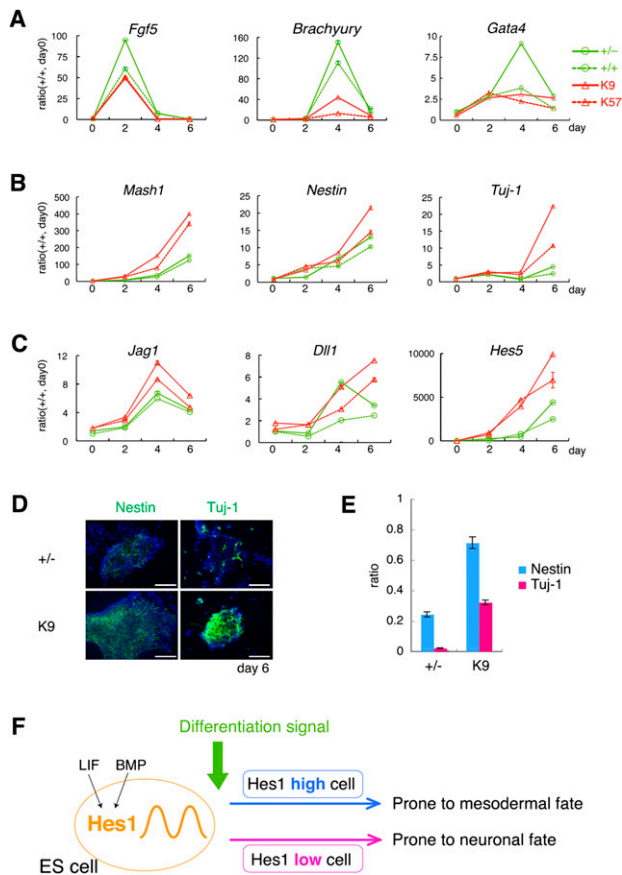
Figure 5. *Hes1*-null ES cells display less heterogeneity in differentiation timing and cell fate choice. Cells were cultured in the N2B27 medium. (*A–C*) mRNA levels of marker genes in the control (+/− and +/+; green) and *Hes1*-null ES cells (K9 and K57; red) during differentiation were analyzed by Q-PCR. Each value was given in the ratio to wild-type cells (+/+) at day 0. (*D*) *Hes1*-null (K9) and parental control cells (+/−) cultured in N2B27 medium were analyzed at day 6 by immunocytochemistory using anti-Nestin and anti-Tuj-1 antibodies (green) with DAPI (blue). Bars, 100 μm. (*E*) Quantification of the ratio of Nestin-positive (blue) and Tuj-1-positive cells (red) of parental (+/−) and *Hes1*-null cells (K9) at day 6. Averages with standard errors were calculated from five fields of confocal images for each cell type. (*F*) Proposed model. Hes1-high and Hes1-low ES cells tend to differentiate into mesodermal and neural cells, respectively.

that *Hes1* and its downstream gene expression dynamically change much faster, over several hours, in ES cells. Hes1-high cells tend to adopt the mesodermal fate whereas Hes1-low cells tend to differentiate into neural cells (Fig. 5F). Furthermore, virtually all *Hes1*-null ES cells differentiated into the neural cells within 6 d under the neural differentiation condition, whereas only subsets of wild-type ES cells did so. Thus, in the absence of *Hes1*, ES cells display less heterogeneity in both the differentiation timing and fate choice, suggesting that the cyclic gene *Hes1* contributes to heterogeneous responses of ES cells. Such rapid cycling of gene expression might be suitable to make multiple cell types even under a single differentiation condition. Hes1 expression also oscillates in neural progenitor cells, but this oscillation seems to contribute to maintenance of the undifferentiated state rather than the diversity in

responses (Shimojo et al. 2008). Thus, it is likely that Hes1 oscillations have different functions in different cell types.

Materials and methods

ES cell lines, culture condition, and real-time imaging

The MG1.19 cell line (Gassmann et al. 1995; Takahashi et al. 2003) was used for the quantification of oscillatory expression and Hes1 knockdown, and the TT2 cell line was used for genetic manipulation. Construction of Hes1 reporter cell lines derived from MG1.19 are described in the Supplemental Material. For real-time imaging, cells were cultured on glass-based dishes in ES medium supplemented with 1 mM luciferin. Luminescence images were captured with 20-min or 10-min exposure and by binning of pixels 8 × 8 or 4 × 4 to increase the signal-to-noise ratio. Strong white dots are derived from cosmic rays. Images collected from CCD camera were analyzed with IMAGE-PRO, as described previously (Masamizu et al. 2006).

Hes1 knockdown

Plasmid vector encoding shRNA under the 7SK promoter (Yoshiura et al. 2007) with polyoma ori for replication (Gassmann et al. 1995) and the puromycin-resistant gene was transfected into MG1.19 ES cells by lipofection, and puromycin-resistant cells were selected. The sequences for Hes1 knockdown were 5′-GCCAATTTGCCTTTCTCATCC-3′ for KD1 and 5′-GTAGAGAGCTGTATTAAGTGA-3′ for KD2. Randomly scrambled sequences were used for control.

Microarray analysis

Total RNA was collected from each cell line after removal of feeder cells and subjected to microarray analysis using GeneChip Mouse Genome 430 2.0 Array (Affimetrix). Data were analyzed by using GCOS (Affimetrix) and were normalized to the parental cells by using Gene Spring (Agilent Technologies). The average of two cell lines are shown in Supplemental Table S1. For ChIP–chip analysis, we prepared two Hes1 knockdown cells for negative control and performed ChIP using rabbit anti-Hes1 antibody as described previously (Ishii et al. 2008). ChIP samples were amplified and hybridized to the mouse promoter 1.0R array and mouse tiling 2.0R array (Affimetrix), and analyzed as described previously (Wendt et al. 2008).

Real-time PCR and immunostaining

Quantification by real-time PCR (Q-PCR) and Western blotting was performed as described previously (Yoshiura et al. 2007). For all real-time PCR analysis, a standard curve was drawn for each primer set using mixtures of cDNA samples. Quantified values of RNA were normalized with those of GAPDH. Primers were listed in Supplemental Table S3. Quantitative analyses were performed at least three times and shown with standard errors. For immunocytochemistry, cells were fixed at 4% PFA on ice, blocked with 0.1% Triton-2% skim milk in PBS, and stained with specific antibodies as described in the Supplemental Material.

Q-PCR of single ES cells

Single cells of the wild-type MG1.19 ES cell line were randomly picked up manually by a glass capillary mouse pipet, and cDNA production and amplification were performed as described previously (Kurimoto et al. 2007). After single-cell cDNA amplification, we performed Q-PCR using specific primers as described previously (Kurimoto et al. 2006; Kawaguchi et al. 2008). Primers are listed in Supplemental Table S3. The copy number of each gene was calculated using Coefficient A and B, which were measured for all primer pairs by using plasmid or mouse genome, and normalized with the spike RNA *Lys* (1000 copies per cell) as described previously (Kurimoto et al. 2006).

Construction of Dll1 and Gadd45g reporters

For *Dll1* reporter plasmid, the promoter region of pDll1-Ub1-Luc (Shimojo et al. 2008) was replaced with a 6-kb *Dll1* promoter including Hes1-binding sites cloned from mouse genome. For *Gadd45g* reporter plasmid, 3-kb *Gadd45g* promoter including Hes1-binding sites and 5′-UTR were placed upstream of and 3′-UTR of *Gadd45g* was placed downstream from ubiquitin (Ub)-nuclear localization signal (NLS)-luc2. Lentiviral vector construction (Miyoshi 2004) is described in the Supplemental Material.

Cell sorting by flow cytometory

ES cells were dissociated by 0.25% trypsin-EDTA, suspended in ES medium and passed through a cell strainer with a pore size of 35μm. Cells were sorted by a FACSAria Cell Sorter (BD Bioscience). MG1.19 wild-type cells were used for negative control. Single cells were gated by forward scatter (FSC), side scatter (SSC), a pulse width parameter of both FSC and SSC. Sorted cells were collected in normal ES medium or N2B27 medium, and cultured on gelatin-coated dish.

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