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Basic helix-loop-helix proteins and the timing of oligodendrocyte differentiation

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SUMMARY

An intracellular timer in oligodendrocyte precursor cells is thought to help control the timing of their differentiation. We show here that the expression of the *Hes5* and *Mash1* genes, which encode neural-specific bHLH proteins, decrease and increase, respectively, in these cells with a time course expected if the proteins are part of the timer. We show that enforced expression of *Hes5* in purified precursor cells strongly inhibits the normal increase in the thyroid hormone receptor protein TRβ1, which is thought to be part of the timing mechanism; it also strongly inhibits the differentiation induced by either mitogen withdrawal or thyroid hormone treatment. Enforced expression of *Mash1*, by contrast, somewhat accelerates the increase in TRβ1 protein. These findings suggest that Hes5 and Mash1 may be part of the cell-intrinsic timer in the precursor cells.

Key words: Hes5, Mash1, Oligodendrocyte, Thyroid hormone receptor, Timer

INTRODUCTION

In many vertebrate cell lineages, precursor cells divide a limited number of times before they stop and terminally differentiate into postmitotic cells. It is unknown what limits cell proliferation and causes the cells to stop dividing and differentiate.

We have been studying the stopping mechanism in the oligodendrocyte cell lineage in the rodent optic nerve (reviewed in Barres and Raff, 1994). Oligodendrocyte precursor cells migrate into the developing nerve from the brain, beginning before birth (Small et al., 1987). After a period of proliferation, they stop dividing and terminally differentiate into oligodendrocytes, which myelinate the axons in the nerve. The first oligodendrocytes appear in the rat optic nerve around the day of birth and then increase in number for the next 6 weeks (Skoff et al., 1976; Miller et al., 1985; Barres et al., 1992).

The normal timing of oligodendrocyte development can be reconstituted in cultures of dissociated perinatal optic nerve cells: as long as the oligodendrocyte precursor cells are stimulated to proliferate by either astrocytes (Raff et al., 1985) or platelet-derived growth factor (PDGF) (Raff et al., 1988), oligodendrocytes begin to appear at the equivalent of the day of birth. Clonal analyses of either single (Temple and Raff, 1986) or purified (Barres et al., 1994) precursor cells isolated from postnatal day 7-8 (P7-8) rat optic nerve suggest that both a cell-intrinsic programme and extracellular signals play important parts in determining when the precursor cells stop dividing and differentiate. In the presence of appropriate signalling molecules, the precursor cells divide up to eight times before they stop and differentiate, and the progeny of an individual precursor cell tend to stop dividing and differentiate at about the same time (Temple and Raff, 1986; Barres et al., 1994). Moreover, when the two daughter cells of an individual precursor cell are separated and cultured on astrocyte monolayers in separate microwells, they tend to differentiate more or less synchronously, suggesting that an intrinsic mechanism operates in the precursor cells to cause them to withdraw from the cell cycle and differentiate after a certain period of time or number of cell divisions (Temple and Raff, 1986). When precursor cells are cultured at 33°C rather than 37°C, they divide more slowly but differentiate sooner, after fewer cell divisions, suggesting that the intrinsic mechanism does not operate by simply counting cell divisions but instead measures elapsed time in some other way (Gao et al., 1997).

At least two kinds of extracellular signals are required for the intrinsic timer to operate normally – the mitogen PDGF (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988) and hydrophobic signals such as thyroid hormone (TH) (Barres et al., 1994; Ibarrola et al., 1996; Ahlgren et al., 1997). In the absence of mitogen, cultured precursor cells prematurely stop dividing and differentiate into oligodendrocytes within 2 days (Noble and Murray, 1984; Temple and Raff, 1985), whether or not hydrophobic signals are present (Barres et al., 1994; Ahlgren et al., 1997). In the presence of mitogen but in the absence of hydrophobic signals, most of the precursor cells in culture tend to keep dividing and do not differentiate for at least 16 days; if TH is added to such cultures after 8 days, however, most of the cells stop dividing and differentiate within 4 days, suggesting that a timing mechanism of some kind continues to operate in the absence of hydrophobic signals (Barres et al.,

1994). These and other (Bögler and Noble, 1994) findings suggest that the intrinsic timer consists of at least two components: a timing component, which measures elapsed time independently of hydrophobic signals such as TH, and an effector component, which stops cell division and initiates differentiation and is regulated by TH. TH also seems to regulate the timing of oligodendrocyte differentiation in vivo (Ibarrola et al., 1996; Ahlgen et al., 1997; Knipper et al., 1998).

The cyclin-dependent kinase (Cdk) inhibitor p27/Kip1 (p27) is apparently one element of the timer and seems to play a part in both the timing and effector components, as both are perturbed in p27-deficient precursor cells (Casaccia-Bonnefil et al., 1997; Durand et al., 1997, 1998). As cell numbers are increased in all organs that have been examined in p27-deficient mice (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996), it seems likely that p27 normally plays a similar role in limiting cell proliferation in many cell lineages. The β TH receptors (TRβs) also seem to be part of the timer, apparently helping maturing precursor cells to respond to TH by withdrawing from the cell cycle and differentiating (Barres et al., 1994; Gao et al., 1998). The levels of both p27 (Durand et al., 1997) and TRβ1 (Gao et al., 1998) progressively increase as the precursor cells proliferate in culture in the presence of PDGF but in the absence of TH; the levels reach plateau values at around the time that most of the precursor cells would normally stop dividing and differentiate were TH present. The increases occur faster at 33°C than at 37°C (Gao et al., 1997, 1998), as might be expected of proteins that play a part in a timer that runs faster at the low temperature (Gao et al., 1997).

We recently provided evidence that the Id4 helix-loop-helix (HLH) protein may also play a part in the timer: Id proteins are thought to act as dominant negative inhibitors of basic HLH (bHLH) proteins that regulate cell-type-specific gene expression during cell commitment and differentiation (reviewed in Norton et al., 1998); and *Id4* expression in oligodendrocyte precursors decreases as the cells proliferate, while *Id4* overexpression inhibits the differentiation of these cells (Kondo and Raff, 2000). Moreover, Wang et al., (1998) have shown that activation of Notch signalling in these cells both induces the expression of *Hes5*, a mammalian homolog of the 'antineural' *Drosophila* bHLH genes *Hairy* and *Enhancer-of-split* (Akazawa et al., 1992), and inhibits differentiation. Together, these findings suggest that bHLH proteins normally regulate oligodendrocyte differentiation.

In the present study, we show that *Hes5* mRNA progressively decreases as the precursors proliferate in vivo and in vitro. Moreover, enforced expression of *Hes5* in purified precursor cells strongly inhibits the normal increase in TRβ1 protein and blocks differentiation triggered by either PDGF withdrawal or TH addition. By contrast, the expression of the proneural gene *Mash1*, a mammalian homolog of *Drosophila achaete-scute* genes (Johnson et al., 1990), progressively increases as oligodendrocyte precursors proliferate in vivo and in vitro. Enforced expression of *Mash1* in purified precursor cells accelerates somewhat the increase in TRβ1 protein that normally occurs in proliferating precursor cells. Together, these findings are consistent with the possibility that the transcriptional regulation of *Hes5* and *Mash1* is part of the intrinsic timer in oligodendrocyte precursor cells and plays a part in regulating both the increase in TRβ1 protein and oligodendrocyte differentiation.

MATERIALS AND METHODS

Animals and chemicals

Sprague/Dawley rats were obtained from the Animal Facility at University College London. Chemicals were purchased from Sigma, except where indicated. Recombinant human PDGF-AA and neurotrophin-3 (NT-3) were purchased from Peprotech.

Preparation of purified precursor cells

Optic nerve cells were prepared from postnatal rats at different ages, and oligodendrocytes and their precursor cells were purified to greater than 99% purity by sequential immunopanning, as described previously (Barres et al., 1992). The purified cells were cultured in poly-D-lysine (PDL)-coated, 6-well, culture dishes (Falcon) or slide flasks (Nunc) in serum-free Dulbecco's Modified Eagle's medium (DMEM) containing bovine insulin (10 μg/ml), human transferrin (100 μg/ml), BSA (100 μg/ml), progesterone (60 ng/ml), putrescine (16 μg/ml), sodium selenite (40 ng/ml), N-acetylcysteine (60 μg/ml), forskolin (5 μM), PDGF-AA (10 ng/ml), NT-3 (5 ng/ml), penicillin and streptomycin (GIBCO) (culture medium). If cultures were maintained for longer than 4 days, half of the medium was replaced every 2 days.

RT-PCR analysis

Cells were harvested by trypsinization and poly(A)$^+$ RNA was prepared using a QuickPrep Micro mRNA Purification kit (Pharmacia Biotech): 1.5 μg of partially purified poly (A)$^+$ RNA was reverse transcribed in 33 μl of reaction mixture, using a First-Strand cDNA Synthesis kit (Pharmacia Biotech). The RT-PCR reaction was carried out in a 50 μl reaction mixture that contained 3 μl of cDNA as template, 1 pM of the specific oligonucleotide primer pair, 10% DMSO (for *Mash1*) and 1.25 units of Taq DNA polymerase. Cycle parameters for *Hes1*, *Hes3*, *Hes5* and *Mash1* were 30 seconds at 94°C, 30 seconds at 63°C and 2 minutes at 72°C for 35 cycles. The cycle parameters for *β-actin* were 15 seconds at 94°C, 30 seconds at 53°C and 1 minute at 72°C for 25 cycles.

The following oligonucleotide DNA primers were synthesized.

For *Hes1*, the 5′ primer was 5′ CCATGCCAGCTGATATAA-TGGAGAAAAA 3′ and 3′ primer was 5′ AATCAGTTCCGCCA-CGGCCTCCA 3′.

For *Hes3*, the 5′ primer was 5′ TTATGGAGAAGAAGCGTCGTG 3′ and 3′ primer was 5′ TTTCACCAGGGCCGCCACACGC 3′.

For *Hes5*, the 5′ primer was TTATGGCCCCAAGTACCGTGGCG and 3′ primer was TTTCACCAGGGCCGCCAGAGGC.

For *Mash1*, the 5′ primer was AACTCGAGATGGAGAGCT-CTGGCAAGATGGA and 3′ primer was AATCGCGATCAGAAC-CAGTTGGTAAAGTCCAG.

For *β-actin*, the 5′ primer was 5′TGGAATCCTGTGGCATCC3′ and the 3′ primer was 5′TCGTACTCCT GCTTGCTG3′.

Full-length rat *Mash1* cDNA was kindly provided by David Anderson. Full-length rat *Hes5* cDNA was amplified by RT-PCR, using pfu Turbo polymerase (Invitrogen) and cloned into a pMOSBlue vector (Amersham Pharmacia Biotech). The *Hes5* sequence was determined using a BigDye Terminator kit and an ABI sequencer (model 310).

Recombinant retrovirus vectors encoding Hes5 or Mash1

To express the *Hes5* and *Mash1* genes efficiently in oligodendrocyte precursor cells and to mark the transfected cells, we made recombinant retrovirus vectors. The gene was inserted into the pBabeG vector, which is based on the pBabe vector (Morgenstern and Land, 1990) but contains the coding sequence for enhanced green fluorescein protein (GFP), driven by the SV40 early promoter. The pBabeG-Hes5 and pBabeG-Mash1 vectors were transfected into Phoenix packaging cells (Kinsella and Nolan, 1996) using LipofectAmine (GIBCO BRL) and the culture supernatant was harvested 3 days after transfection. To concentrate the recombinant

virus, 10 ml of culture supernatant was centrifuged at 20,000 revs/minute for 2 hours, as described in Current Protocols (Ausubel et al., 1992). The virus pellet was suspended in 1 ml of culture medium and 0.2 ml of the virus solution was used to infect oligodendrocyte precursor cells purified from postnatal day 6 (P6) rats. The cells were cultured with virus overnight, washed and grown in culture medium with PDGF for 2 days. To induce differentiation into oligodendrocyte, the cells were then cultured either with PDGF and TH for 5 days or without PDGF for 3 days. To induce differentiation into type-2 astrocytes (Raff et al., 1983), the cells were then cultured in 15% fetal bovine serum (FBS) for 3 days. In other cases, purified newborn (postnatal day 0, P0) or P6 precursor cells were infected and cultured in PDGF without TH for a total of 5 or 7 days and then immunostained for TRβ1 protein (as described below).

Immunocytochemistry

To determine whether retrovirus-infected cells expressing GFP had differentiated into oligodendrocytes, cells growing in slide flasks were fixed with 4% paraformaldehyde for 15 minutes at room temperature and then stained with monoclonal anti-galactocerebroside (GC) antibody (Ranscht et al., 1982; supernatant, diluted 1:3) to detect oligodendrocytes (Raff et al., 1978) or with the A2B5 monoclonal antibody (Eisenbarth et al., 1979; ascites fluid, diluted 1:100) to detect oligodendrocyte precursors (Raff et al., 1983). The monoclonal antibodies were detected with Texas-Red-conjugated goat anti-mouse IgG or IgM, respectively (Jackson ImmunoResearch, diluted 1:100), as previously described (Gao et al., 1998). To examine the level of TRβ1 protein in *Hes5*- or *Mash1*-overexpressing cells, cells were fixed as above, treated with 50% normal goat serum and 0.1% Triton X-100, and then stained with a monoclonal anti-TRβ1 antibody (StressGen, diluted 1:100), followed by biotin-conjugated goat anti-mouse IgG (Amersham, diluted 1:100) and then Texas-Red-conjugated streptavidin (Amersham, dilution 1:100). To determine whether retrovirus-infected cells expressing GFP had differentiated into type-2 astrocytes (Raff et al., 1983), the cells were fixed and stained for TRβ1 as just described, except that a rabbit anti-glial fibrillary acidic protein (GFAP) antiserum (Pruss, 1979; diluted 1:100) was used instead of the anti-TRβ1 antibody and biotin-conjugated goat anti-rabbit IgG (Amersham, diluted 1:100) was used to detect the anti-GFAP antibodies.

Mash1 protein was detected as described previously (Shah et al., 1994). In brief, cells were fixed in paraformaldehyde as above, treated with 10% normal goat serum and 0.1% NP-40, and then stained with a monoclonal anti-Mash1 antibody (Shah et al., 1994; supernatant, diluted 1:1), using a mouse ABC staining system (Santa Cruz).

The stained slides were mounted in Citifluor mounting medium (CitiFluor, UK), sealed with nail varnish, and the intensity of fluorescence was quantified in a Bio-Rad MRC 1000 confocal laser-scanning fluorescence microscope as previously described (Durand et al., 1997; Gao et al., 1998).

RESULTS

Hes5 mRNA decreases as oligodendrocyte precursors proliferate in vitro and in vivo

To determine whether *Hes* genes might play a part in timing oligodendrocyte differentiation, we examined the expression of three mammalian *Hes* genes – *Hes1*, *Hes3* and *Hes5* – in purified P0 oligodendrocyte precursor cells. Using RT-PCR, we found that *Hes1*, *Hes3* and *Hes5* mRNAs were all expressed in the cultured precursor cells (not shown). The expression of *Hes1* and *Hes3* did not decrease when the precursor cells differentiated into oligodendrocytes (not shown), and we did not study these genes further. The expression of *Hes5*, however, decreased as purified P0 precursor cells proliferated in culture in PDGF without TH and became undetectable when the cells were induced to differentiate into oligodendrocytes by PDGF withdrawal for 2 days (Fig. 1A). *Hes5* mRNA also decreased as oligodendrocyte precursor cells proliferated in vivo: when *Hes5* mRNA levels were examined in precursor cells freshly purified from P0, P7 and P9 optic nerves, the levels were seen to decrease progressively with age (Fig. 1B).

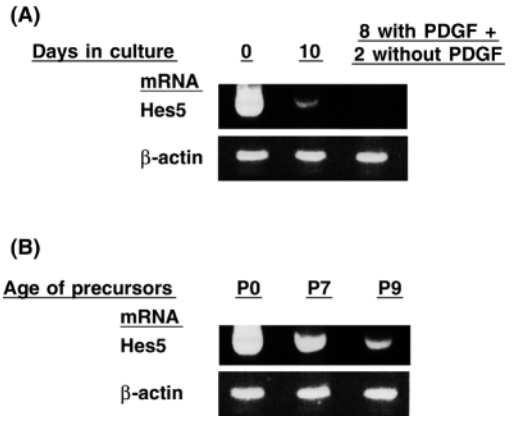

Fig. 1. The level of *Hes5* mRNA in oligodendrocyte precursor cells, assayed by RT-PCR, decreases with time in vitro and in vivo. (A) Purified P0 precursor cells were cultured for 0 or 10 days in the presence of PDGF and the absence of TH. In some wells, the cells were induced to differentiate into oligodendrocytes by removing PDGF at 8 days and continuing the culture for 2 days. (B) Freshly purified P0, P7 and P9 precursor cells were studied without culture. *β-actin* mRNA was assayed as a control. The experiments in this and the following figures were repeated at least once, with similar results.

These results are consistent with the possibility that a progressive decrease in *Hes5* expression may be part of the intrinsic timer in oligodendrocyte precursor cells. Unfortunately, suitable anti-Hes5 antibodies were not available to examine the expression of Hes5 protein.

Mash1 mRNA and protein increase as oligodendrocyte precursor cells proliferate in vitro and in vivo

To determine if Mash1 is expressed in oligodendrocyte precursor cells, we first used RT-PCR to look for *Mash1* mRNA. As shown in Fig. 2A, *Mash1* mRNA was expressed weakly in freshly prepared P0 precursor cells, and its expression level gradually increased after 5 days and 10 days of culture in PDGF without TH; expression remained high when precursor cells differentiated into oligodendrocytes in response to PDGF withdrawal for 2 days. Using a monoclonal anti-Mash1 antibody (kindly provided by David Anderson), we could readily detect Mash1 protein in the nucleus of both P0 precursor cells cultured for 10 days (Fig. 3C) and oligodendrocytes (Fig. 3E); the protein was barely above background in P0 precursor cells cultured for only 1 day (Fig. 3A) and was a little greater after 5 days (Fig. 3B). These results indicate that Mash1 mRNA and protein increase in parallel as precursor cells proliferate in culture and remain high in newly formed oligodendrocytes, at least for a day or two.

To determine whether *Mash1* mRNA also increases as oligodendrocyte precursor cells proliferate in vivo, we used RT-

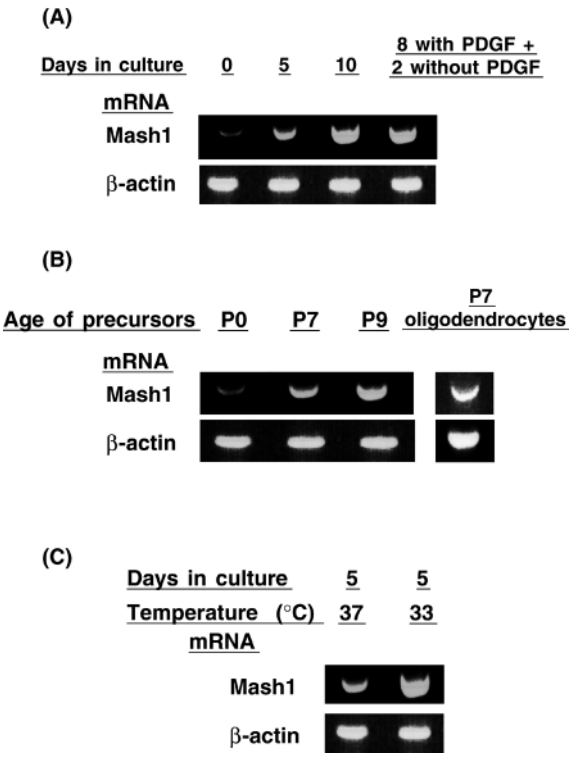

Fig. 2. The level of *Mash1* mRNA in oligodendrocyte precursor cells increases with time in vitro and in vivo and increases faster in vitro at 33°C than at 37°C. (A) Purified P0 precursor cells were cultured as in Fig. 1A. (B) Freshly purified P0, P7 and P9 precursor cells and P7 oligodendrocytes were studied without culture. (C) P0 precursor cells were cultured for 1 day at 37°C and then for a further 4 days at either 33°C or 37°C.

PCR to study its expression in freshly purified precursors from P0, P7 and P9 rat optic nerve. As shown in Fig. 2B, the level of *Mash1* mRNA gradually increased with age. Furthermore, *Mash1* mRNA was readily detected in oligodendrocytes freshly purified from P7 rat optic nerve. These results suggest that *Mash1* expression progressively increases as precursor cells proliferate in vivo and is still expressed in newly formed oligodendrocytes, at least transiently.

To determine if *Mash1* expression increases faster at 33°C than at 37°C, we analyzed Mash1 mRNA and protein in purified P0 precursors cultured for 5 days at the two temperatures. Both *Mash1* mRNA (Fig. 2C) and Mash1 protein (Fig. 3B,D) increased faster at the lower temperature.

Taken together, these results are consistent with the possibility that a progressive increase in Mash1 is part of the cell-intrinsic timer in oligodendrocyte precursor cells and that the faster increase at 33°C may be part of the reason that the timer runs faster at 33°C than 37°C.

Overexpression of *Hes5* or *Mash1* influences the normal increase in TRβ1 protein

TRβ1 (Gao et al., 1998) and TRβ2 (Barres et al., 1994) proteins may be part of the timer that regulates the timing of oligodendrocyte differentiation. The promoter region of the *TRβ* gene contains an N-box (CACNAG) and E-boxes (CANNTG), which are potential binding sites for Hes5 and Mash1 proteins, respectively (Sakurai et al., 1992; reviewed in Kageyama et al., 1995, 1997). Thus these bHLH transcription factors are potential regulators of *TRβ* expression in oligodendrocyte precursors and may be partly responsible for the progressive increase in the TRβ proteins as these cells proliferate. To examine whether overexpression of *Hes5* or *Mash1* can alter *TRβ1* expression, we infected purified P6 or P0 precursor cells overnight with the BabeG-Hes5 or BabeG-Mash1 retrovirus vectors, respectively, or with the control BabeG virus, which only encodes GFP. To examine the effects of *Hes5* or *Mash1* overexpression, we cultured the cells for an additional 7 or 5 days, respectively. We then stained the cells with a monoclonal anti-TRβ1 antibody and quantified the staining with a confocal laser-scanning fluorescence microscope as previously described (Durand et al., 1997; Gao et al., 1998), identifying infected cells by their expression of GFP. TRβ1 protein was high in all of the BabeG (Fig. 4B) and BabeG-Mash1 (Fig. 4C)-infected cells and was significantly lower in BabeG-Hes5-infected cells (Fig. 4A). As shown in Table 1, the average level of TRβ1 in BabeG-infected cells was about 7-fold higher than in BabeG-Hes5-infected cells and about 1.5-fold lower than in BabeG-Mash1-infected cells (Table 1). Thus overexpression of *Hes5* markedly inhibited the normal increase in TRβ1 protein that occurs as the precursor cells proliferate in culture, consistent with the possibility that the

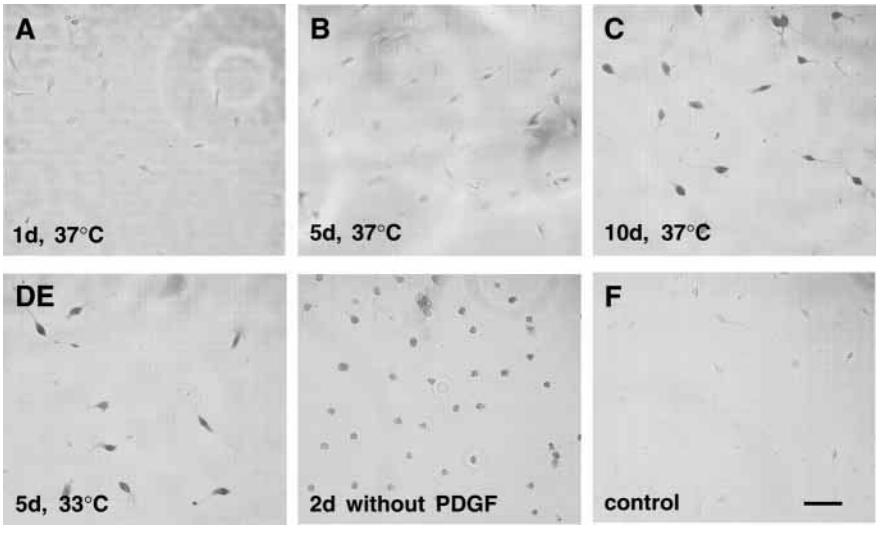

Fig. 3. Immunoperoxidase staining of Mash1 protein in oligodendrocyte precursor cells. Purified P0 precursor cells were cultured in PDGF without TH at 37°C for various periods (A-C) or for 1 day at 37°C and a further 4 days at 33°C (D). (E) Oligodendrocyte differentiation was induced by PDGF withdrawal for 2 days after 8 days of culture. (F) Background staining of cells cultured as in C but stained without Mash1 primary antibody. Scale bar, 50 μm.

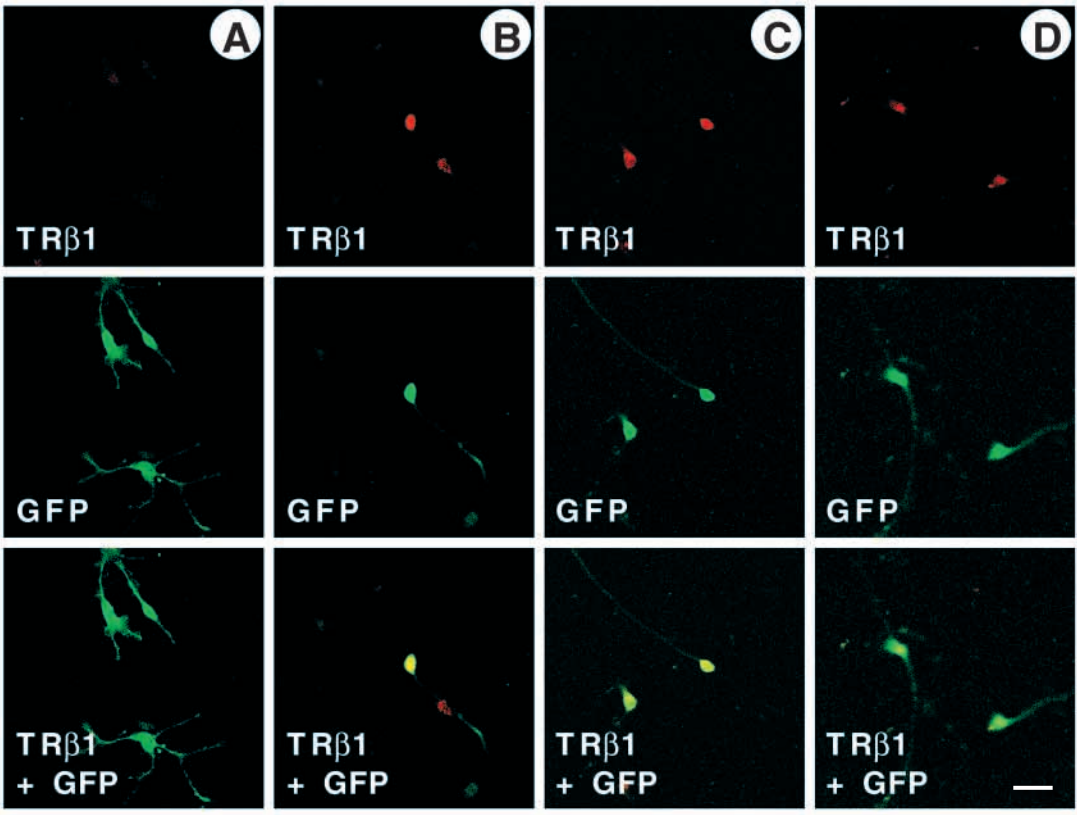

Fig. 4. Effects of *Hes5* and *Mash1* transgene expression on the levels of TRβ1 protein detected by immunofluorescence and confocal microscopy. Purified P6 (A,B) or P0 (C,D) precursor cells were infected overnight with either the BabeG-Hes5 virus (A) or the BabeG-Mash1 virus (C), respectively. As a control, both P6 and P0 precursor cells were also infected with the BabeG virus (B,D). The cells were then cultured for another 7 days in PDGF without TH and then fixed and stained for TRβ1 (shown in red in the upper panels). The virus-infected cells were detected by the expression of GFP (shown in green in the middle panels). The fused images of TRβ1 staining and GFP are shown in the bottom panels. Scale bar, 25 μm.

Hes5 protein normally restrains *TRβ1* expression in these cells. Although the effect of *Mash1* expression was small, it was reproducible and statistically significant, consistent with the possibility that the Mash1 protein may normally help stimulate *TRβ1* expression.

Overexpression of *Hes5* prevents the normal increase in Mash1 protein

In neuronal development, the expression of *Mash1* is negatively regulated by Hes1 and Hes5 proteins (Ishibashi et al., 1995; de la Pompa et al., 1997). To examine if Hes5 can negatively regulate *Mash1* expression in oligodendrocyte precursor cells, we infected purified P4 precursor cells overnight with either the BabeG or BabeG-Hes5 retrovirus and cultured them for another 5 days in PDGF without TH. We then stained the cells with a monoclonal anti-Mash1 antibody. Mash1 protein was high in more than 90% of the BabeG-infected cells (Fig. 5A) and was much reduced in about 70% of the BabeG-Hes5-infected cells (Fig. 5B), consistent with the possibility that Hes5 normally regulates *Mash1* expression in oligodendrocyte precursor cells.

Overexpression of *Hes5* inhibits oligodendrocyte differentiation

Overexpression of Hes proteins has been shown to inhibit the differentiation of various kinds of cells, including neurons and muscle cells (Ishibashi et al., 1994; de la Pompa et al., 1997; Jarriault et al., 1998; Ohtsuka et al., 1999). To determine if *Hes5* overexpression inhibits oligodendrocyte differentiation, we infected purified P6 precursor cells overnight with the BabeG-Hes5 or BabeG retrovirus vectors and cultured them for another 2 days in PDGF without TH. To induce the cells to differentiate into oligodendrocytes, we then cultured the cells either in the absence of PDGF for 3 more days or in PDGF and TH for 5 more days. The cells were then stained with anti-GC antibody to identify oligodendrocytes (Raff et al., 1978; Ranscht et al., 1982) and with the A2B5 antibody to identify precursor cells (Raff et al., 1983). In both cases, most of the BabeG-Hes5-infected cells failed to differentiate: they remained $A2B5^+$ (Fig. 6B) and GC^- (Fig. 6C) and retained the bipolar morphology characteristic of oligodendrocyte precursor cells (Fig. 6B and 6C). By contrast, most of the BabeG-infected cells had become $A2B5^-$ (not shown) and GC^+ (Fig. 6A) and had acquired the typical appearance of oligodendrocytes (Fig. 6A). In cultures where oligodendrocyte differentiation was induced by PDGF

Table 1. Effect of the *Mash1* or *Hes5* transgene expression on the level of TRβ1 protein expression

Experiment 1		Experiment 2	
Retroviral vector	Intensity of TRβ1 staining ($\times 10^4$ pixels)	Retroviral vector	Intensity of TRβ1 staining ($\times 10^4$ pixels)
BabeG	9.4±1.3	BabeG	7.7±1.6
BabeG-Hes5	1.4±0.9*	BabeG-Mash1	11.2±2.5*

Purified P6 (experiment 1) or P0 (experiment 2) precursor cells were infected with the retrovirus vector overnight, cultured for 7 (experiment 1) or 5 (experiment 2) more days in PDGF without TH, and then fixed and stained for TRβ1. The intensity of TRβ1 staining in GFP^+ cells was measured using a confocal microscope. 50 cells in three independent cultures in single experiments were assessed for each value, which is expressed as mean ± s.d. The experiments were repeated twice with similar results.

* indicates a significant difference ($P<0.001$ for experiment 1 and $P<0.05$ for experiment 2) compared to the result with BabeG, when analyzed by Student's *t*-test.

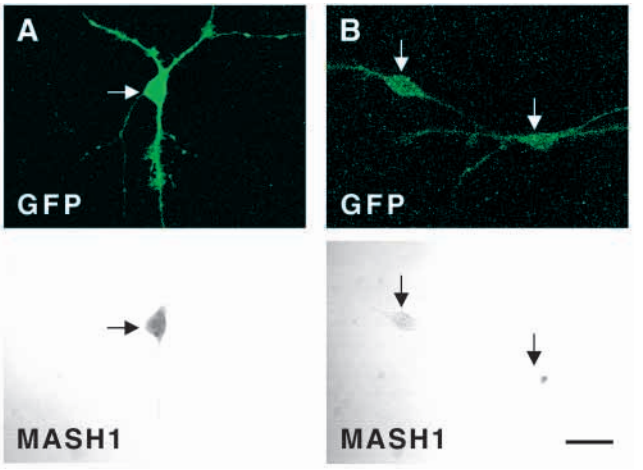

Fig. 5. Effect of *Hes5* transgene expression on the level of Mash1 protein detected by immunoperoxidase staining. Purified P6 precursor cells were infected overnight with either the BabeG control virus (A) or the BabeG-Hes5 virus (B). They were then cultured in PDGF without TH for another 5 days and then immunoperoxidase stained with anti-Mash1 antibody (lower panels). The virus-infected cells were detected by the expression of GFP (green in the upper panels). About 100 cells of each type were analyzed and about 70% of the BabeG-Hes5-infected cells showed significantly reduced staining compared to the BabeG-infected cells. Scale bar, 25 μm.

Table 2. Inhibitory effect of the *Hes5* transgene on oligodendrocyte differentiation

	PDGF withdrawal		TH addition	
	% A2B5$^+$	% GC$^+$	% A2B5$^+$	% GC$^+$
BabeG	18±4	81±3	21±4	73±5
BabeG-Hes5	75±6	23±3	77±4	19±4

Purified P6 precursor cells were infected with the retrovirus vector, cultured in PDGF without TH for 2 days and then cultured either in the absence of PDGF for 3 days or in PDGF with TH for 5 days to induce oligodendrocyte differentiation. The cells were then stained for A2B5 or GC. The proportion of GFP$^+$ cells that were A2B5$^+$ or GC$^+$ are shown as mean ± s.d. of 3 cultures. The experiment was repeated twice with similar results.

withdrawal, more than 80% of BabeG-infected cells differentiated into GC$^+$ oligodendrocytes, whereas less than 25% of BabeG-Hes5-infected cells did so (Table 2). In cultures where differentiation was induced by TH addition, more than 70% of BabeG-infected cells differentiated into GC$^+$ oligodendrocytes, whereas less than 20% of BabeG-Hes5-infected cells did so (Table 2). These results suggest that the overexpression of *Hes5* inhibits oligodendrocyte differentiation induced by either PDGF withdrawal or TH addition and raise the possibility that the gradual decrease in Hes5 in developing precursor cells may play a part in the normal timing of oligodendrocyte differentiation.

Oligodendrocyte precursor cells can be induced to differentiate into type-2 astrocytes by culturing them in ≥10%

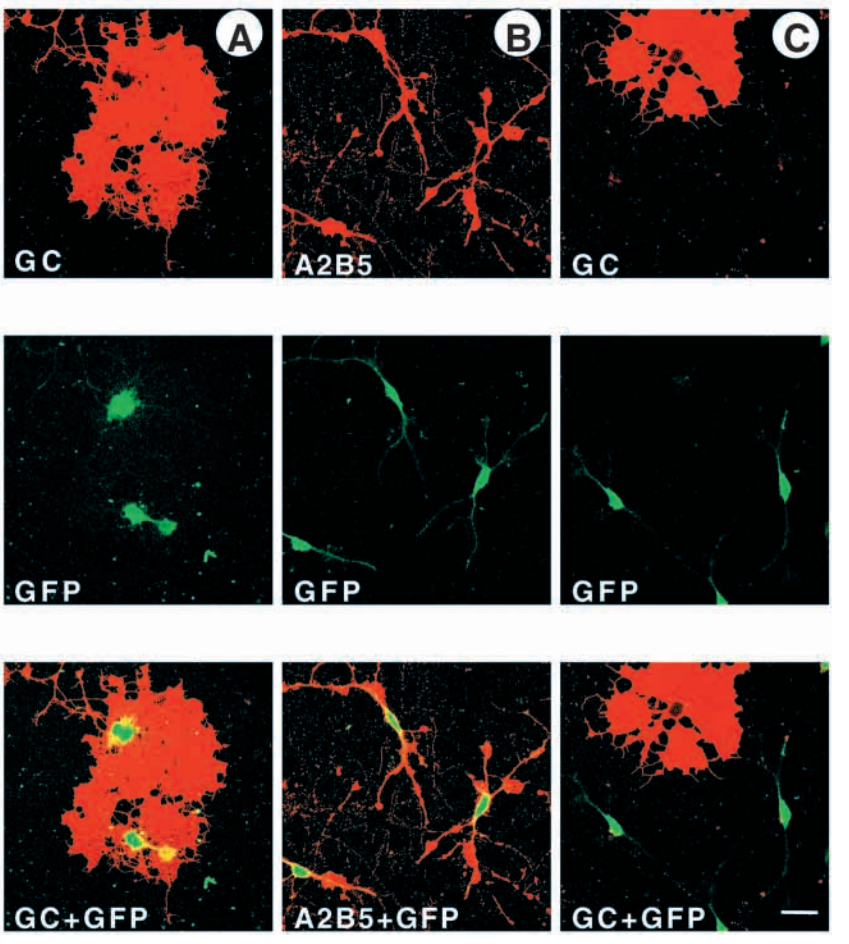

Fig. 6. Effect of *Hes5* transgene expression on oligodendrocyte differentiation, assessed by immunofluorescence. Purified P6 precursor cells were infected overnight with either the BabeG control virus (A) or the BabeG-Hes5 virus (B,C). They were then cultured in PDGF without TH for another 2 days and then in the absence of PDGF for a further 3 days to induce the cells to differentiate into oligodendrocytes. The cells were then immunostained with anti-GC (A,C) or A2B5 monoclonal antibodies (B). The antibody staining is shown in red in the upper panels. The virus-infected cells were detected by the expression of GFP (green in the middle panels). The fused images of the antibody staining and GFP are shown in the bottom panels. Note that the GC$^+$ cells in (C) are GFP$^-$ and therefore presumably uninfected. Scale bar, 25 μm.

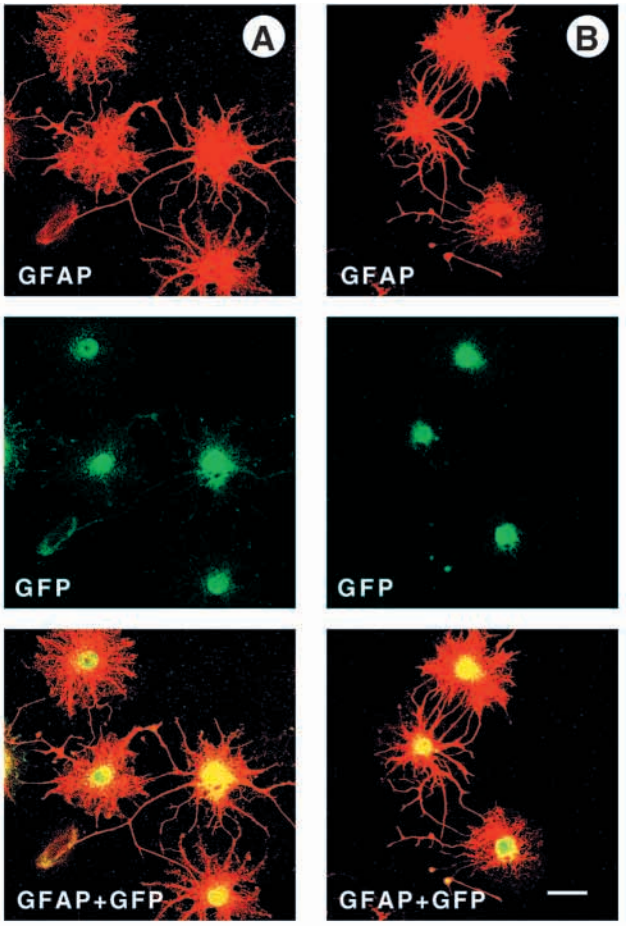

Fig. 7. Effect of *Hes5* transgene expression on type-2 astrocyte differentiation, assessed by immunofluorescence. Purified P6 precursor cells were infected overnight with either the BabeG control virus (A) or the BabeG-Hes5 virus (B). They were then cultured with PDGF for another 2 days and then in 15% FBS for a further 3 days to induce them to differentiate into type-2 astrocytes. The cells were immunostained with an anti-GFAP antiserum (red in the upper panels), and the virus-infected cells were detected by the expression of GFP (green in the middle panels). The fused images of GFAP staining and GFP are shown in the bottom panels. Scale bar, 25 μm.

FBS (Raff et al., 1983). To determine if the overexpression of *Hes5* inhibits type-2 astrocyte differentiation, we infected P6 precursor cells with either BabeG or BabeG-Hes5 retrovirus, cultured them for 3 days in 15% FBS, and then stained them for GFAP, a marker of astrocytes (Bignami et al., 1972; Raff et al., 1978). Both types of infected cells readily differentiated into type-2 astrocytes (Fig. 7), indicating that *Hes5* overexpression did not appreciably inhibit type-2 astrocyte differentiation.

To determine if *Mash1* overexpression can accelerate oligodendrocyte differentiation, we infected purified P0 precursor cells overnight with the BabeG-Mash1 or BabeG retrovirus vectors, cultured them for 2 days in PDGF without TH and then for another 5 days in PDGF and TH to allow the cells to differentiate under the control of their intrinsic timer (Barres et al., 1994). The cells were then stained with anti-GC antibody to identify oligodendrocytes and with the A2B5 antibody to identify the precursor cells. *Mash1* overexpression did not accelerate oligodendrocyte differentiation under these conditions: about 20% of the infected cells differentiated into oligodendrocytes in both cases (not shown).

DISCUSSION

Both positively acting and negatively acting bHLH proteins, which bind to E-boxes and N-boxes, respectively, help control the development of various cell types (reviewed in Kageyama et al., 1995). In *Drosophila*, for example, a balance between the proneural bHLH proteins Achaete-scute, Atonal and Daughterless, and the 'antineural' bHLH proteins Hairy, Enhancer-of-split and Extramacrochaetae help control neural development (Klambt et al., 1989; Ellis et al., 1990; Skeath and Carroll, 1991; Campuzano and Modolell, 1992). Neural-specific homologues of these proteins, including the proneural protein Mash1 and the 'antineural' proteins Hes1 and Hes5, help control neural development in mammals (reviewed in Kageyama et al., 1995; Lee, 1997). We have studied the expression of *Mash1* and *Hes5* in cells of the oligodendrocyte lineage to see if their expression patterns and the effects of their overexpression are consistent with their playing a part in regulating the timing of differentiation of oligodendrocyte precursor cells.

Hes5

The Hes family of bHLH proteins can bind to and antagonize the action of proneural bHLH proteins such as Mash1 (Akazawa et al., 1992; Sasai et al., 1992; Tietze et al., 1992). They can also bind to N-box elements in DNA and recruit co-repressor proteins to inhibit proneural gene transcription (Fisher et al., 1996; Grbavec and Stifani, 1996). Both Hes1 and Hes5 have been shown to be highly expressed in developing mammalian brain and to be downregulated as development proceeds: they are expressed in neural precursor cells in the ventricular zone, for example, and are lost as the cells differentiate and migrate away from this zone (Akazawa et al., 1992; Sasai et al., 1992). Both *Hes1* and *Hes5* genes are activated by Notch signalling (Jarriault et al., 1998; Wang et al., 1998; Ohtsuka et al., 1999), which is thought to inhibit neural differentiation in the ventricular zone and elsewhere (Akazawa et al., 1992; Ishibashi et al.,1994). When either *Hes1* or *Hes5* is inactivated by targeted gene disruption, neural differentiation is accelerated, and the acceleration is enhanced if both genes are inactivated (Tomita et al., 1996; Ohtsuka et al., 1999).

Notch has been shown to be expressed by oligodendrocyte precursor cells (Wang et al., 1998) and, when it is activated by the binding of the Notch ligand Delta, it induces the expression of *Hes5* and inhibits oligodendrocyte differentiation (Wang et al., 1998). Our finding that the overexpression of *Hes5* can on its own inhibit oligodendrocyte differentiation induced by either mitogen withdrawal or TH addition strongly suggests that Hes5 protein is an important effector of the Notch-mediated inhibition. We show here that *Hes5* mRNA progressively decreases as developing oligodendrocyte precursor cells proliferate in vivo and in vitro and is no longer detectable when the precursors differentiate into oligodendrocytes. It seems likely that the progressive decrease in Hes5 protein plays a part in regulating when the precursor

cells differentiate. As we cannot detect *Hes5* mRNA by RT-PCR in newly formed oligodendrocytes, it seems likely that the decrease in mRNA reflects, in part at least, a decrease in transcription. The decrease in *Hes5* expression in vivo may reflect a decrease in Notch signalling, as both Notch and its ligand Jagged 1 progressively decrease with age in the developing rat optic nerve (Wang et al., 1998). Decreased Notch signalling, however, seems unlikely to be the only explanation for the decrease of *Hes5* mRNA in our cultures of purified oligodendrocyte precursor cells: the decrease occurs very slowly (over days), even though there should be little Notch signalling in our cultures, as the precursors do not express Notch ligands (Wang et al., 1998). We can still detect *Hes5* mRNA, for example, after 10 days in culture. It may be, therefore, that at least part of the progressive decrease in *Hes5* mRNA reflects on intrinsically timed decrease in *Hes5* transcription. The *Hes5* promoter region contains multiple N-boxes (Takebayashi, et al., 1995), raising the possibility that the Hes5 protein may help downregulate its own expression, although the gradual decrease in Hes5 expression over many days suggests a more complex mechanism of control.

Whatever its mechanism, the progressive decrease in *Hes5* expression would be expected to increase the transcriptional activity of Mash1, and perhaps other proneural bHLH proteins. As the promoter of the *TRβ* gene contains E boxes, the increase in these proneural bHLH proteins could well play a part in the progressive increase the TRβ proteins (Barres et al., 1994; Gao et al., 1998) in developing oligodendrocyte precursor cells. Our finding that the expression of a *Hes5* transgene in these cells prevents the increase in both Mash1 and TRβ1 protein is consistent with this possibility. As *Hes5* mRNA does not decrease faster at 33°C than at 37°C (T. K., unpublished observations), however, its decrease presumably does not contribute to the faster increase in *Mash1* expression (this study) or the faster operation of the intrinsic timer at the lower temperature (Gao et al., 1997).

The progressive decrease in *Hes5* expression may also allow other proneural bHLH proteins to promote the maturation and differentiation of oligodendrocyte precursor cells. Two closely related bHLH genes, called *olg-1* (or *oligo1*) and *olg-2* (*oligo2*), that are preferentially expressed in oligodendrocyte lineage cells have recently been reported (Lu et al., 2000; Zhou et al., 2000). The timing of their expression suggests that they may be involved in the specification of oligodendrocyte precursor cells, as well as in their differentiation.

Mash1

Mash1 is required for the development of certain types of mammalian neurons, including noradrenergic neurons and primary olfactory neurons (Guillemot et al., 1993; Blaugrund et al., 1996; Hirsch et al., 1998). It is expressed in the precursor cells of sympathetic neurons, for example, and is downregulated after the cells differentiate (Lo et al., 1991; Guillemot et al., 1993; Jasoni and Reh, 1996). In mice in which the *Mash1* gene has been inactivated by targeted gene disruption, all noradrenergic neurons fail to develop (Sommer et al., 1995; Hirsch et al., 1998).

In a preliminary report, Wang et al. (1999) independently detected *Mash1* expression in oligodendrocyte lineage cells. We show here that Mash1 mRNA and protein increase in developing oligodendrocyte precursor cells as they proliferate in vivo and in vitro. Moreover, both the mRNA and protein increase faster at 33°C than at 37°C. One possible explanation for the faster increase at 33°C is that the low temperature may decrease the rate of synthesis of Mash1 protein or *Mash1* mRNA (or both) less than it decreases their rates of degradation. Overexpression of *Mash1* slightly, but consistently, accelerates the rate at which TRβ1 protein increases in purified precursor cells proliferating in culture, suggesting that Mash1 can directly or indirectly regulate *TRβ1* expression in these cells. Overexpression of *Mash1*, however, did not detectably accelerate oligodendrocyte differentiation when the precursor cells were cultured in PDGF and TH. It seems that the increase in Mash1 (and the resulting small increase in TRβ1) achieved in our experiments, is not enough on its own to accelerate oligodendrocyte differentiation. Nonetheless, it is possible that Mash1 normally helps stimulate TRβ1 expression and may be part of the mechanism that helps control the timing of oligodendrocyte differentiation. It will be important to study the timing of oligodendrocyte development in Mash1-deficient mice to test this possibility.

In summary, our findings raise the possibility that the two bHLH genes that we have studied, *Mash1* and *Hes5*, may be part of the cell-intrinsic timing mechanism that regulates oligodendrocyte precursor cell maturation (Gao and Raff, 1997) and differentiation (Temple and Raff, 1986; Barres et al., 1994). Moreover, enforced expression of *Hes5*, and to a lesser extent *Mash1*, alters the expression of *TRβ1* in the direction that supports this possibility. A major challenge is to discover how these cell-intrinsic changes in gene expression, which occur gradually over many days in purified precursors proliferating in a relatively constant environment, are controlled.

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