The potential to induce glial differentiation is conserved between *Drosophila* and mammalian glial cells missing genes

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Summary

***Drosophila glial cells missing* (*gcm*) is a key gene that determines the fate of stem cells within the nervous system. Two mouse *gcm* homologs have been identified, but their function in the nervous system remains to be elucidated. To investigate their function, we constructed retroviral vectors harboring *Drosophila gcm* and two mouse Gcm genes. Expression of these genes appeared to influence fibroblast features. In particular, mouse *Gcm1* induced the expression of astrocyte-specific Ca^{2+}-binding protein, S100β, in those cells. Introduction of the mouse *Gcm1* gene in cultured cells from embryonic brains resulted in the induction of an astrocyte lineage. This effect was also observed by in utero injection of retrovirus harboring mouse *Gcm1* into the embryonic brain. However, cultures from mouse *Gcm1*-deficient mouse brains did not exhibit significant reductions in the number of astrocytes. Furthermore, in situ hybridization analysis of mouse *Gcm1* mRNA revealed distinct patterns of expression in comparison with other well-known glial markers. The mammalian homolog of *Drosophila gcm*, mouse *Gcm1*, exhibits the potential to induce gliogenesis, but may function in the generation of a minor subpopulation of glial cells.**

Key words: *glial cells missing* (*gcm*), Glial development, Astrocyte, Retrovirus

Introduction

Recently, our understanding of the molecular mechanisms governing mammalian neurogenesis has increased substantially through the isolation and characterization of the genes homologous to those playing important roles in *Drosophila* neurogenesis. Many genes are conserved between flies and mammals, and they function in similar ways in both species. One of the most important issues in developmental neurobiology is the cell fate determination by neural stem cells. In *Drosophila*, neuronal and glial fates are controlled by the *glial cells missing* gene, *gcm*, in the manner of binary fate decision (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). In a *gcm* mutant, glial cells are converted to neurons, whereas ectopic expression of *gcm* in neurons causes neuron-to-glia transformation. Biochemical analyses have revealed that Gcm has the characteristics of a transcription factor with an N-terminal DNA-binding domain and a C-terminal transactivation domain (Akiyama et al., 1996; Schreiber et al., 1997; Schreiber et al., 1998).

Two mammalian *gcm* homologs (*Gcm1* and *Gcm2*, previously known as *Gcma* and *Gcmb*) have been identified in mice, rats and humans (Akiyama et al., 1996; Altshuller et al., 1996; Kammerer et al., 1999; Kanemura et al., 1999; Kim et al., 1998). Sequence homology between *Drosophila* and mammalian Gcm proteins is restricted to the N-terminal region, which contains the DNA-binding domain. In agreement, all Gcms bind the same DNA sequence, (A/G)CCCGCAT (Akiyama et al., 1996; Schreiber et al., 1998). When mouse *Gcm1* was ectopically expressed in the *Drosophila* nervous system, formation of additional glial cells was observed (Kim et al., 1998; Reifegerste et al., 1999). This indicates that mouse Gcm1 is functionally similar to *Drosophila* Gcm. The sequence conservation and the interchangeable activity initially led us to predict that mammalian Gcm plays a role in gliogenesis. Contrary to this expectation, mammalian Gcm genes were expressed in the nervous system at extremely low levels, detectable only by sensitive RT-PCR (Altshuller et al., 1996; Basyuk et al., 1999; Kammerer et al., 1999; Kanemura et al., 1999; Kim et al., 1998). The main sites of *Gcm1* and *Gcm2* expression are the placenta (Basyuk et al., 1999; Kim et al., 1998) and parathyroid glands (Kim et al., 1998), respectively. Targeted disruption of the mouse *Gcm1* gene in mice results in a severe defect in labyrinth formation in the placenta, which leads to embryonic lethality between embryonic day 9.5 and 10 (E9.5-10) (Anson-Cartwright et al., 2000; Schreiber et al., 2000). No abnormalities were detected in the embryo proper at least until death. By contrast, mouse *Gcm2*-targeted mice exhibit a selective loss of the parathyroid glands, but no abnormalities were reported in the nervous system (Gunther et al., 2000). These findings raise the speculation that mammalian Gcm genes have a biological role other than in gliogenesis.

In the present study, we have elucidated the function of mammalian Gcm genes in the central nervous system by employing retrovirus-mediated gene expression. In developing brain cells, mouse *Gcm1* induced astrocyte cell fate and

suppressed neuronal cell fate both in vitro and in vivo. However, the expression pattern of the mouse Gcm1 gene was distinct from those observed for the early astrocyte markers, such as GLAST and brain-lipid-binding protein (BLBP) (Shibata et al., 1997; Hartfuss et al., 2001). Moreover, brain cultures from mouse *Gcm1*-deficient mice did not display a significant reduction in the number of astrocytes. Taken together, we speculate that mouse *Gcm1* may functionally contribute towards the generation of a minor subpopulation of glial cells.

Materials and methods

RT-PCR

PolyA$^+$ RNA was prepared from embryonic day 16 (E16) ICR mouse (Japan SLC) placenta and brain using the QuickPrep *Micro* mRNA Purification Kit (Amersham Biosciences). Total RNA from mouse brains at different developmental stages was isolated with TRIzol Reagent (Invitrogen). PolyA$^+$ or total RNA (1 μg) was converted to cDNA using AMV RNA polymerase (Seikagaku, Tokyo). Primers for mouse *Gcm1* (5′-gccatgcgcaacaccaacaaccacaat-3′, 5′-atcatgctcgccttt-tggactggaa-3′), mouse *Gcm2* (5′-agacagcacgcaggacgaggatgctgt-3′, 5′-ggccttgtcacagatggctggcctcag-3′), β-actin (5′-taccaactgggacgacatgga-gaa-3′, 5′-gctcgaagtctagagcaacatagc-3′), S100β (5′-ttgccctcattgatg-tcttccaccag-3′, 5′-cttgtgaccctcatgtctgttgcaga-3′) were designed to recognize sequences located on different exons. PCR reactions (Perkin Elmer) were performed for 20-35 cycles (94°C for 30 seconds; 68°C for 30 seconds; 72°C for 1 minute; plus a final extension 72°C for 10 minutes). PCR products were run on a polyacrylamide gel and visualized by ethidium bromide staining. To analyze the developmental profile, real-time PCR was performed with LightCycler (Roche). The cDNA corresponding to 50 ng of RNA was used in a 20 μM reaction containing 3 mM $MgCl_2$, 0.5 μM of each primer and LightCycler SYBR Green I mix (Roche). Reaction conditions included an initial denaturation at 95°C for 8 minutes, followed by 50 cycles of 95°C for 5 seconds, 68°C for 5 seconds and 72°C for 12 seconds. Fluorescence was detected at the end of each cycle to monitor the amount of PCR product. Amplification of specific transcripts was confirmed by a final melting curve profile and gel electrophoresis. Levels of mouse Gcm transcripts at each stage were calibrated by comparison with β-actin values.

Retroviral vectors

The *gcm* and mouse *Gcm* cDNAs (Akiyama et al., 1996; Hosoya et al., 1995) were cloned into a retroviral vector, pTY20E$^+$ (Ikeda et al., 1997), which has an internal ribosome entry sequence (IRES) followed by the *lacZ* gene. A control vector contains a *neo*^{r} gene instead of a *gcm* gene. Retroviral plasmid DNA was transfected into retroviral packaging cells, ΨMP34 (Yoshimatsu et al., 1998), using LipofectAMINE plus Reagent (Invitrogen). ΨMP34 was derived from the NIH3T3 cell line and modified to provide high-titer virus (Yoshimatsu et al., 1998). Several days after transfection, β-galactosidase-positive cells were collected by fluorescent activated cell sorter, FACS Vantage (BD Biosciences) using a fluorogenic substrate, fluorescein di-β-galactopyranoside according to a method (FDG-FACS) previously reported (Nolan et al., 1988). Fluorescein di-β-galactopyranoside was synthesized as described (Ikenaka et al., 1990). FACS was repeated until stable transformants were obtained. Supernatants containing virus were collected and titered on NIH3T3 cells. Concentration and medium change of viral solutions were performed by centrifugation at 6000 ***g*** at 4°C for 16 hours (Bowles et al., 1996). For the histochemical detection of β-galactosidase, cultures were fixed with 0.5% glutaraldehyde and then incubated with 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) solution, including 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 and 1 mM $MgCl_2$ in PBS at 37°C overnight. Rabbit polyclonal anti-mouse Gcm1 (peptide antibody against DVKLPQNVKTTDWFQEWPDS) and anti-mouse Gcm2 (antibody against GST fusion mouse Gcm2 protein; a gift from Dr Masato Nakafuku, University of Tokyo) were used to detect the expressed mouse Gcm proteins in each stable transformant. Retroviral vectors containing alkaline phosphatase (Ikeda et al., 1997) were used as an internal control for culture conditions. For colony formation assays, NIH3T3 cells (1×10^4) were seeded onto 3.5 cm dishes and infected with recombinant virus with 8 μg/ml polybrene (Sigma-Aldrich) the next day. Five days later, cultures were stained with X-gal and the numbers of cells in clusters were counted.

Primary cell culture and retrovirus-mediated gene expression

Hemispheres were dissected from ICR mouse embryos (E12.5) and mechanically triturated in medium by passing through a heat-polished Pasteur pipette. Dissociated cells were washed with DMEM/F12 medium (DF; Sigma) containing 10% fetal bovine serum (FBS; EQUITECH-BIO) and plated on eight-well slides coated with polyethyleneimine (Sigma) at a density of 1×10^5 cells/cm^2. The following day, cells were incubated with retroviruses (~1×10^6 cfu/cm^2) with 1 μg/ml polybrene for 4 hours, and then the medium was replaced with serum-free DF medium containing N2 supplement (Invitrogen). Two or three days after infection, cultures were fixed with 2% paraformaldehyde (PFA) and 0.02% glutaraldehyde in PBS, and incubated in a X-Gal solution at 37°C for 4-8 hours. Following the X-gal reaction, cells were stained with anti-MAP-2 (Sigma) or anti-GFAP (DAKO) antibodies. For O4 staining, the cells were incubated with O4 antibody (a gift from Dr Steven E. Pfeiffer, University of Connecticut) before fixation. The cells were subsequently incubated with biotinylated goat anti-mouse IgM antibody (Vector Laboratories), followed by Vectastain ABC-peroxidase detection (Vector Laboratories). For S100β$^+$ staining, the culture was fixed with 4% PFA and incubated with anti-β-gal (Cappel) and anti-S100β (Sigma) antibodies, followed by detection with secondary antibodies conjugated to Alexa^{488} or Alexa^{594} (Molecular Probes). Detection of alkaline phosphatase activity was performed as described previously (Ikeda et al., 1997).

Prenatal retrovirus injections and histochemical analysis

Pregnant mice containing E13 embryos were anesthetized with Nembutal (Abbott), and after midline laparotomy, fetal heads were transilluminated with a fiber optic source, and the location of the lateral ventricles was identified. A retroviral vector carrying the *lacZ* reporter gene was injected into the lateral ventricle of each embryo. Approximately 1 μl of retroviral suspension at a titer of 1×10^9 cfu/ml with 0.05% Fast Green (Sigma) was injected through a glass capillary. The injected fetuses were born normally and allowed to survive for up to 2 months. P24 mice were used for histological analysis. They were perfused with a fixative solution containing 4% paraformaldehyde, 0.2% glutaraldehyde, 0.02% NP-40 and 0.1 M phosphate buffer (pH 7.4). The brains were removed from the skulls and washed in PBS containing 1 mM $MgCl_2$ at 4°C three times. Subsequently, the brains were incubated in a X-gal solution. After the X-gal reaction, brains were sectioned serially in the coronal plane at 100 μm using a Vibratome, or at 30 μm using a Cryostat for further staining with GFAP and MAP2 antibodies.

Analysis of mouse *Gcm1* deficient mice

Brain cells from E9.5 mouse *Gcm1* mutant mice (T.H. and Y.H, unpublished) were cultured according to Kitani's method (Kitani et al., 1991) with some modifications. Briefly, heads were dissected and incubated with 0.25% trypsin for 7 minutes at 37°C. After addition of FBS, cells were triturated in a DF medium supplemented with 10% FBS by pipeting and plated on a non-coated 3.5 cm dish. After incubation for 4 hours, untouched cells were collected and cultured again on a non-coated 3.5 cm dish. The following day, untouched cells

were collected and plated on a PEI-coated eight-well glass slide. Their embryonic bodies were used for PCR genotyping. Primers were: *lacZ*, (5′-attaggtccctcgaaggaggttcac-3′, 5′-tgagtttatgttccaccgtgcagc-3′); and mouse Gcm1, (5′-aacgactgactggttccaggagtgg-3′, 5′-ggccttgtcacagatg-gctggcctcag-3′). After 3 or 5 days, cultures were fixed with 4% PFA and incubated in blocking solution (PBS with 3% normal goat serum and 0.1% Triton X-100) for 1 hour at room temperature. Cells were then stained with anti-MAP2, S100β and GFAP antibodies, followed by detection with secondary antibodies conjugated with Alexa^{488} or Alexa^{594} (Molecular Probes). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

In situ detection of mouse *Gcm1* mRNA in the embryonic brain

In situ hybridization was performed as previously described (Kagawa et al., 1994). For preparation of embryonic brain tissues, perfusion fixation using 4% paraformaldehyde was performed. After fixation, tissues were embedded in paraffin wax, sectioned at 8 μm and put on APS-coated slides (Matsunami). Sections were treated with 0.2% pepsin for 2-3 minutes at 37°C, and hybridized with 100 ng/ml riboprobe in 50% formamide, 20 mM Tris-HCl (pH 7.5), 600 mM NaCl, 1 mM EDTA, 10% dextran sulfate, 200 μg/ml yeast tRNA, 1×Denhardt's solution, 0.25% SDS at 50°C overnight. The sections were washed with 2×SSC containing 50% formaldehyde at 50°C for 20 minutes, followed by 0.2×SSC at 50°C for 20 minutes twice. Digoxigenin (DIG)-labeled riboprobes were synthesized from linearized mouse *Gcm1* plasmids (359 bp fragment of mouse Gcm1 cDNA; position 1260-1619, GenBank Accession Number D88612) using the DIG RNA labeling kit (Roche). DIG probes were visualized by alkaline phosphatase-conjugated anti-DIG antibody and NBT/BCIP reaction (Roche). Sense probe was used as a control.

Results

Developmental profiles of mouse Gcm gene expression in the brain

PolyA$^+$ RNA was prepared from the mouse brain and placenta at embryonic day 16 (E16). The primers were designed to hybridize to sequences from different exons to avoid amplification from contaminating genomic DNA. Consistent with previous studies (Basyuk et al., 1999; Kim et al., 1998), mouse *Gcm1*-mRNA was abundant in the placenta (Fig. 1A). We detected mouse *Gcm1* and mouse *Gcm2* transcripts by single 35-cycle PCR (Fig. 1A), which indicated that mouse Gcm mRNA was not a very rare species in embryonic brains, contrary to previous findings (Altshuller et al., 1996; Basyuk et al., 1999; Kammerer et al., 1999; Kanemura et al., 1999; Kim et al., 1998). We next examined Gcm mRNA expression levels in developing brains from E12 to adult mice by real-time PCR. The expression of mouse *Gcm1* and mouse *Gcm2* at each age was calibrated against β-actin expression and is plotted in Fig. 1B. Expression of mouse *Gcm1* in the brain peaked at E12, and decreased thereafter (Fig. 1B). In comparison to mouse *Gcm1*, the expression of mouse *Gcm2* was higher and remained nearly constant throughout development (Fig. 1B). Mouse Gcm2 was also expressed in the placenta.

Expression of mouse Gcm genes influences fibroblast characteristics

We investigated the function of mouse Gcm genes in the developing brain by the retrovirus-mediated gene expression technique. Each mouse Gcm cDNA was subcloned into pTY20E$^+$ (Ikeda et al., 1997), upstream of the IRES and bacterial β-galactosidase. The retroviral vectors were introduced into retroviral packaging cells, ΨMP34 (Yoshimatsu et al., 1998) by lipofection. Expression of mouse Gcm proteins in stable ΨMP34 transformants was confirmed by staining with an antibody specific for each protein (Fig. 2A,B). As expected based on the features of transcriptional factors, Gcm proteins were localized to the nuclei. Gcm gene transfection frequently induced abnormal morphologies including an enlarged cell body and quite long processes with several knots (Fig. 2D,E). This effect was not observed when truncated mouse Gcm2 containing the DNA-binding domain but not the transactivating domain was expressed (Fig. 2F). This suggests that the abnormal morphologies were caused by the transcriptional activation mediated by mouse Gcm.

After the transfection, stable transformants were collected by cell sorting using fluorogenic substrates of β-galactosidase. However, concentration of Gcm transformants proved to be difficult. After collecting β-galactosidase$^+$ cells, growth of

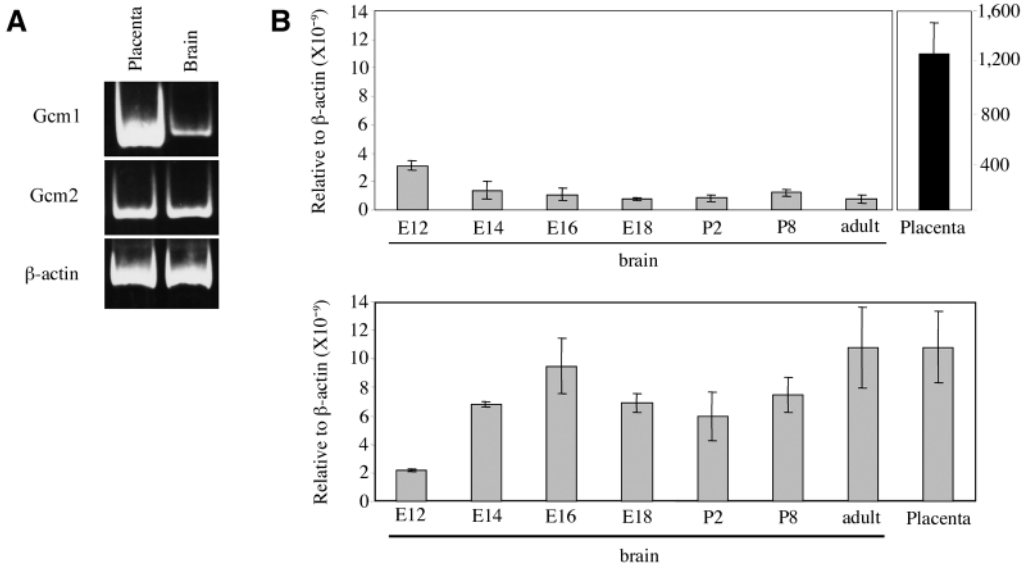

Fig. 1. Developmental profiles of Gcm gene expression in the mouse brain. (A) Messenger RNA was prepared from E16 placenta and brain, and used for RT-PCR analysis. Primers specific for mouse *Gcm1*, mouse *Gcm2* and β-actin were designed to recognize positions on different exons. (B) Total RNA was prepared from different developmental stages of the mouse brain, and used for quantification of mouse Gcm gene expression by real-time PCR analysis. Bars represent the relative mouse Gcm transcript levels normalized against β-actin transcript levels.

Gcm-transformants was much slower in comparison with control transformants and a population of X-gal$^+$ cells gradually decreased during culture. We speculated that the ectopic expression of Gcm genes causes growth retardation in fibroblast cells. To assess this, we examined the colony formation abilities of Gcm-expressing cells. Fibroblast cells were infected with the control (Fig. 2G), mouse *Gcm1* (Fig. 2H), mouse *Gcm2* (Fig. 2I) or *Drosophila gcm* (Fig. 2J) retroviral vector at an extremely low titer to produce X-gal$^+$ cells as a colony among X-gal$^-$ cells. In the control experiment, the number of X-gal$^+$ cells in a colony ranged from 1 to 160, with an average around 40 cells (Fig. 2K). However, the colony size of mouse *Gcm1*, mouse *Gcm2* and *Drosophila gcm* transduced cells shifted to a smaller size in comparison with the control, and the respective averages sizes were below 20 cells (Fig. 2K). The truncated mouse *Gcm2*, however, did not exhibit such an effect (data not shown).

We speculated that these cellular effects are caused by transactivation of certain genes by Gcm, and thus examined expression of several glial genes by RT-PCR. Among several genes, the expression of S100β, a Ca^{2+}-binding protein expressed in astrocytes, was highly upregulated in mouse *Gcm1*-transduced cells (Fig. 2L). Significant induction of S100β was also observed in mouse *Gcm2*-transduced cells. Expression of S100β was minimal in the control-transduced (Fig. 2L) or non-transduced (data not shown) fibroblast cells. Expression and induction of GFAP or PLP mRNAs were not detected in these fibroblast cells. GLAST, which is expressed in the early glial lineage, was highly expressed in non-transduced fibroblast cells and a change in expression level owing to Gcm gene transduction was not evident (data not shown).

Effect of *gcm*-expression on cultured embryonic brain cells

In order to determine whether the mouse Gcm genes are involved in glial cell fate determination in the developing nervous system, we forced the expression of mouse *Gcm1* or mouse *Gcm2* in cells cultured from E12 mouse brains. Following retroviral infection, cells were cultured in a chemically defined medium for 3 days. The cells were fixed and stained with X-Gal, followed by staining with neuronal marker MAP2, astrocyte marker GFAP or oligodendrocyte marker O4. Under these culture conditions from early embryonic brains, only a small number of cells (<1%) were

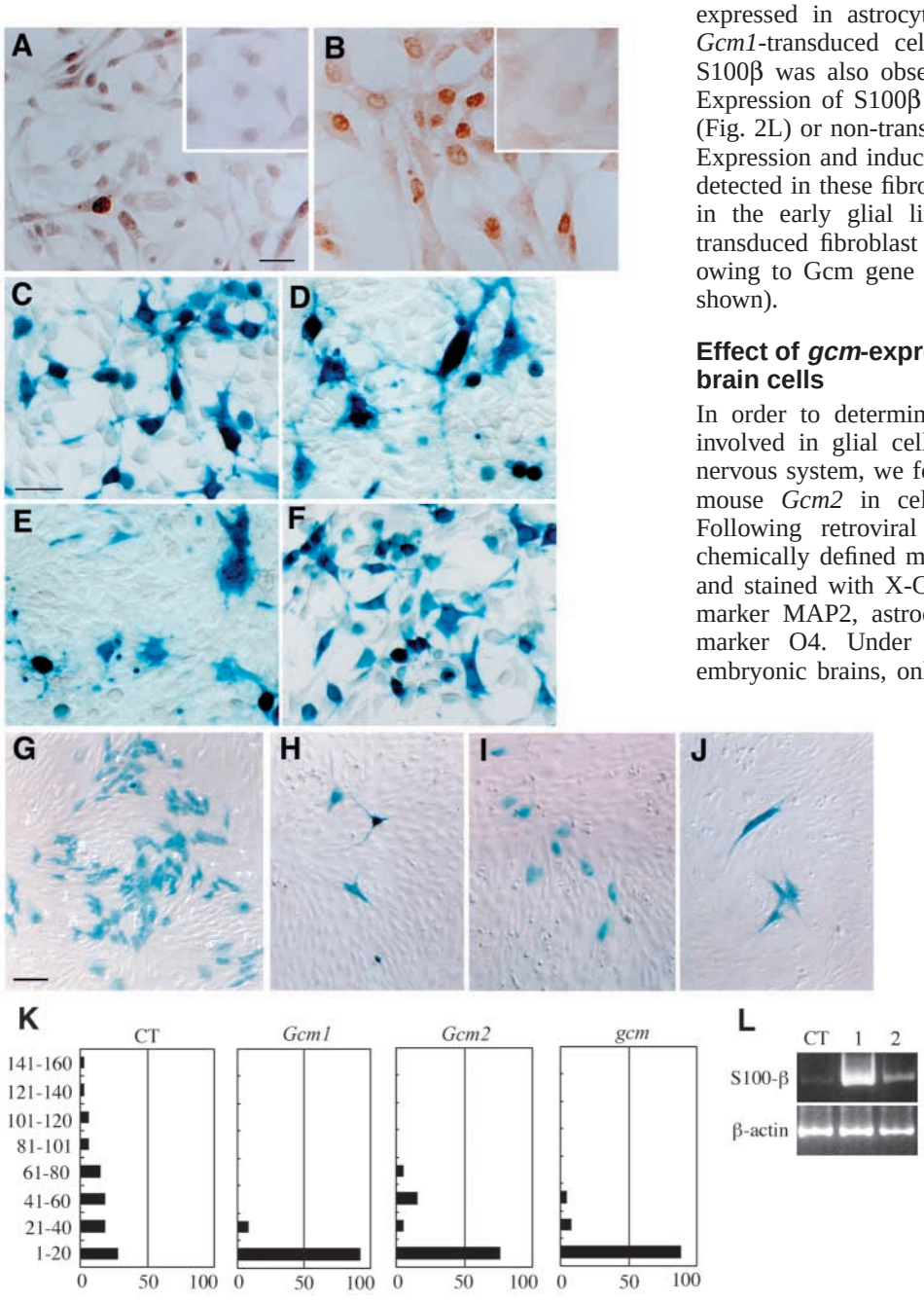

Fig. 2. Effects of the Gcm genes in fibroblast cells. (A,B) Staining of mouse Gcm1 (A) and mouse Gcm2 (B) proteins expressed in each stable transformant. The proteins were localized in nuclei. The top right panels show negative controls in which the first antibodies were omitted. (C-F) Gcm gene expression leads to morphological changes in fibroblast cells. Cells were transfected with control (C), mouse *Gcm1* (D), mouse *Gcm2* (E) or truncated mouse *Gcm2* (F) retroviral vectors and cultured for 3 days. Truncated mouse Gcm2 contains the DNA-binding domain but not the transactivating domain. The cells were subsequently stained with X-gal. (G-K) Fibroblast cells were transduced with control (G), mouse *Gcm1* (H), mouse *Gcm2* (I) or *Drosophila gcm* (J) viruses at low titers and cultured for 6 days. The cells were stained with X-gal (G-J) and the X-gal$^+$ cell number in a cluster was counted. More than 100 colonies for each viral transduction were examined and the distribution of colony size was plotted (K). (L) Total RNA was prepared from stable transformants of control (CT), mouse *Gcm1* (a) and mouse *Gcm2* (b), and used for RT-PCR analysis of S100β and β-actin gene expression. Induction of S100β expression by mouse *Gcm1* and mouse *Gcm2* was observed in fibroblast cells. Scale bars: 25 μm in A,B; 50 μm in C-F; 50 μm in G-J.

GFAP$^+$ after 3 days, although many cells (~30%) were MAP2$^+$ (data not shown). An additional 2 days in culture led to the appearance of many GFAP$^+$ cells in the culture. In control experiments, 3% of the transduced cells were GFAP$^+$ (Fig. 3A,H), but mouse *Gcm1*-expression increased this percentage to more than 30% (Fig. 3B,H). Conversely, 13% of the transduced cells were MAP2$^+$ in the control (Fig. 3D,I), whereas mouse *Gcm1*-expression decreased this to 3% (Fig. 3E,I). These results suggest that mouse *Gcm1* induces the astrocyte lineage while suppressing the neuronal lineage. However, mouse *Gcm2* transduction exhibited no significant differences in comparison with the control (Fig. 3C,F). With regard to the oligodendrocyte lineage, we could not detect O4-positive cells after 3 days culture (data not shown). An additional 3 days in culture led to the appearance of O4-positive cells, but most of the transduced cells were still O4 negative and we were unable to detect any significant effects (data not shown). Induction of GFAP$^+$ cells by mouse *Gcm1* was also observed in the culture after 2 days (Fig. 3G), when GFAP$^+$ cells seldom exist in the control cultures. It was noteworthy that the only mouse *Gcm1*-transduced X-gal$^+$ cells became GFAP$^+$ and the surrounding cells were negative (Fig. 3G). Furthermore, the percentage of GFAP$^+$ cells in transduced cells after 2 days already reached 25%. This indicates that the induction of GFAP$^+$ cells by mouse *Gcm1* was prompt.

We next examined whether another astrocyte-specific protein, S100β, was induced in mouse *Gcm1*-transduced cells. About 20% of transduced cells in the control were already S100β$^+$ at 3 days culture (Fig. 3J,K,N). These results are consistent with the fact that S100β is expressed earlier in the astrocyte lineage than GFAP and in oligodendrocyte lineage (Richter-Landsberg and Heinrich, 1995; Rickmann and Wolff, 1995). Expression of mouse *Gcm1* led to 70% of transduced cells becoming S100β$^+$ (Fig. 3L-N). This indicates that mouse *Gcm1* induces glial lineage and suppresses the neuronal lineage in the primary brain cell culture.

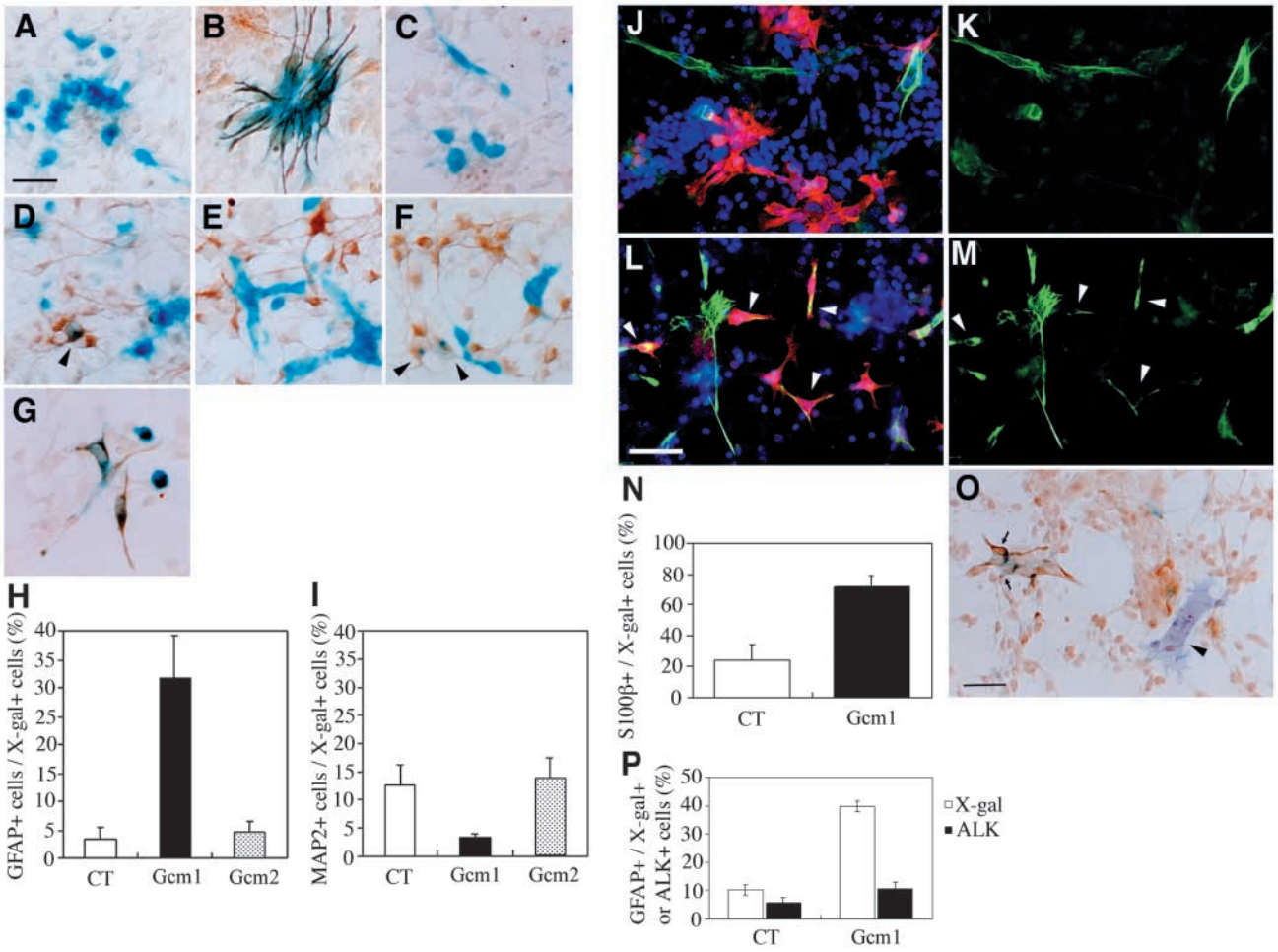

Fig. 3. Effect of the Gcm genes in cultured brain cells. (A-G) Primary cultured cells from E12 mouse hemispheres were transduced with control (A,D), mouse *Gcm1* (B,E,G) or mouse *Gcm2* (C,F) retrovirus and cultured in chemically defined medium for 3 days (A-F) or 2 days (G). The cells were stained with X-Gal, followed by staining with anti-GFAP (A-C,G) or anti-MAP2 (D-F) antibodies. GFAP$^+$ (H) or MAP2$^+$ (I) cells in X-gal$^+$ cells after 3 days culture were counted. (J-N) After control (J,K) or mouse *Gcm1* (L,M) transduction, brain cells were stained with both β-galactosidase (red) and S100β (green) antibodies. J and L are merged images. Arrowheads show overlapped signals in mouse *Gcm1* transduction. The percentage of overlap was plotted in N. (O,P) Brain cells were infected with control *lacZ* or mouse Gcm1-*lacZ* virus together with virus harboring alkaline phosphatase (ALK). *lacZ*$^+$ and ALK$^+$ cells were visualized by X-gal-staining (arrows) and NBT-staining (arrowheads), respectively. They were further stained with anti-GFAP antibodies (brown). Cells double-stained for X-gal and anti-GFAP Ab or ALK and anti-GFAP Ab were counted (P). Scale bars: 30 μm in A-G; 40 μm in J-M,O.

Although longer cultures of transduced cells were attempted, this proved difficult because of gradual cell death caused by mouse *Gcm1* transduction (data not shown). This may be attributed to the misexpression and/or overexpression of mouse *Gcm1*. The cell death of many cells may exhibit negative influences on other cells in culture. We were afraid that such unfavorable conditions in the mouse Gcm gene-transduced culture promoted astrocyte differentiation. To exclude this possibility, we performed mouse *Gcm1-lacZ* viral infections together with alkaline phosphatase (ALK) virus as an internal control (Fig. 3O,P). No significant differences were observed in the appearance of GFAP$^+$ cells in ALK$^+$ cells in culture between the control and mouse *Gcm1* experiment (Fig. 3P). This indicated that the increase of GFAP$^+$ cells was directly induced by mouse *Gcm1* expression, not by detrimental culture conditions after massive cell death.

Retrovirus-mediated expression of mouse *Gcm1* in vivo

To address whether mouse *Gcm1* transduction induces glial lineage in vivo, we performed in utero injection of retroviruses into developing mouse brains. Concentrated viral stocks (1×10^9 cfu/ml) of control and mouse *Gcm1* viruses were prepared and injected into the lateral ventricle of E13 brains using glass capillaries. The brains were then fixed at P24, stained with X-gal, and sectioned serially in a coronal plane at 100 μl using a Vibratome. Fig. 4 shows the section of forebrain injected with control (CT) viruses where morphologies of X-gal$^+$ cells were suggestive of neurons and astrocytes. Neuron-like cells seemed to exhibit a small clear cell body while astrocyte-like cells have a large obscure cell body. To confirm this classification, the cells were double-labeled with X-gal and a neuronal marker, NeuN (Fig. 4C,D), or an astrocyte marker, GFAP (Fig. 4E,F) antibody. Among 73 X-gal$^+$ cells morphologically classified as astrocytes, 72 cells (98.6%) were GFAP$^+$ (Fig. 4E,F), and none (0%) were MAP2$^+$ (Fig. 4D). As reported previously, astrocytes in the white matter or near pia mater exhibited strong immunoreactivity for GFAP (Fig. 4E), while gray matter astrocytes exhibited much weaker staining (Fig. 4F), yet they were identifiable under our staining conditions. By contrast, among 147 X-gal$^+$ cells morphologically classified as neurons, 109 cells (74.1%) were NeuN$^+$ cells (Fig. 4C), and none (0%) were GFAP$^+$. Based on this classification, we scored cell types in the neocortex of P24 brains infected with control or mouse *Gcm1* virus at E13. mouse *Gcm1* expression led to a significant increase in the number of astrocytes, and decrease in the number of neurons, in comparison with the control (Fig. 2G). Expression of mouse *Gcm1* was also shown to effectively promote the generation of astrocyte lineage cells in vivo.

Analysis of mice with targeted disruption of the mouse Gcm1 gene

To directly address the function of mouse *Gcm1* in cell fate determination, we studied the *Gcm1* mutant mice. Because the homozygous mice die too early (~E9.5) to directly assess astrocyte development in vivo, we employed the method of a whole head culture from E9.5 mice (Kitani et al., 1991) to assess the ability to generate astrocytes. In this culture, fibroblasts and other cohesive cells were removed by panning onto the non-coated dish. After several days in culture, the cells were stained with MAP2, S100β or GFAP antibodies. Many MAP2$^+$ and S100β$^+$ cells appeared in the culture from both wild-type and mutant mice after 3 days in culture. At this point, GFAP$^+$ cells were not detected yet. After 2 more days in culture, many GFAP$^+$ cells appeared in the culture from both wild-type and mutant mice. We examined three wild-type (data not shown), four heterozygous and three homozygous mice (Fig. 5), but no significant differences in the number of GFAP$^+$ cells were detected among them. This indicates that the mouse *Gcm1* defect did not affect the generation of the major population of astrocytes.

Distribution of mouse *Gcm1* mRNA in the developing brain

To understand the physiological roles of mouse *Gcm1* in gliogenesis, we attempted to analyze mouse *Gcm1* expression in the early developing brain by in situ hybridization, in spite of the previous report describing difficulty in detecting the

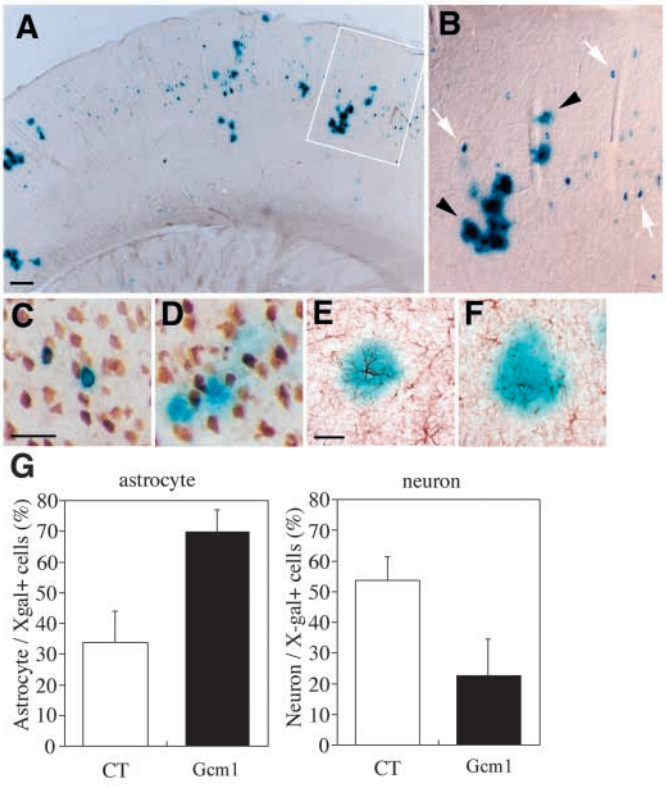

Fig. 4. Effect of mouse Gcm1 expression during gliogenesis in vivo. Vibratome sections of P24 mouse forebrain that was injected with β-gal-harboring retrovirus at E13 were stained with X-gal (A). B is a higher magnification of a box in A. The observed morphologies of infected cells were suggestive of neurons (arrows) and astrocytes (arrowheads). Neuron-like cells have a small clear cell body while astrocyte-like cells have a large obscure cell body. To confirm this classification, the cells were further stained (brown) with a neuronal marker, NeuN (C,D), or an astrocyte marker, GFAP (E,F). Using these criteria, the lineage of cells infected with CT or mouse *Gcm1* retrovirus was examined (G). For mouse *Gcm1*-transduced cells, 69.9±7.2% of cells (n=2, 725 cells scored in total) were astrocytes while 33.6±10.2% (n=2, 1742 cells scored in total) of the cells were astrocytes for CT transduction. Scale bars: in A, 150 μm for A and 60 μm for B; in C, 25 μm for C,D; in E, 50 μm for E,F.

signal (Kim et al., 1998). After vigorous pepsin-treatment, mouse *Gcm1* mRNA was detectable in the forebrain at E14. The signals were dispersed in the ganglionic eminence (Fig. 6A,B) and thalamus (Fig. 6A). These positive cells appeared round and small, and their distribution pattern was distinct from well-known early astrocyte/radial glial markers, such as GLAST or BLBP (Shibata et al., 1997; Hartfuss et al., 2001) (data not shown). Expression of mouse *Gcm1* was confirmed in labyrinth layer of placenta using the same probe (Fig. 6C). Sense probe was used as negative control (Fig. 6D).

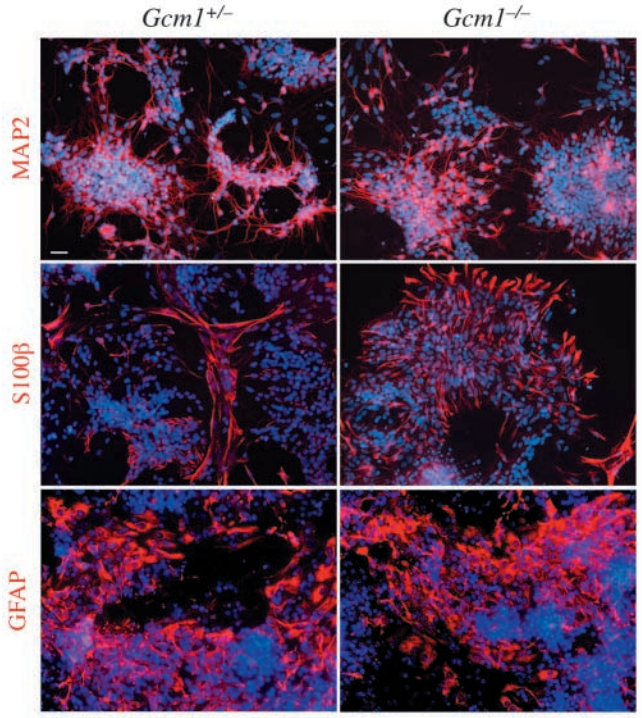

Fig. 5. Cultures from mouse *Gcm1*-deficient mice. Heads were dissected from E9.5 mouse Gcm1-heterozygote or homozygote mice and cultured according to Kitani et al. (Kitani et al., 1991). The cells were stained with anti-MAP2 or S100β antibody after 3 days or with anti-GFAP antibody after 5 days. There were no significant differences in the appearance of GFAP$^+$ cells among the cultures from mouse *Gcm1*-heterozygote or homozygote mice. Scale bar: 30 μm.

Discussion

Although *Drosophila gcm* has a clear role in gliogenesis (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996), the function of its mammalian counterparts in the nervous system has not yet been clarified. Ectopic expression of mouse *Gcm1* in the developing nervous system of *Drosophila* led to the transformation of presumptive neurons into glial cells (Kim et al., 1998; Reifegerste et al., 1999). In addition, *Drosophila* Gcm and mouse Gcm1 proteins exhibited similar DNA-binding specificity and transactivation potential (Akiyama et al., 1996; Schreiber et al., 1998). In spite of their functional conservation, mouse *Gcm1* and mouse *Gcm2* are mainly expressed in organs other than the nervous system, placenta (Basyuk et al., 1999; Kim et al., 1998) and parathyroid glands (Kim et al., 1998), and are detectable only via sensitive RT-PCR in the nervous system (Altshuller et al., 1996; Basyuk et al., 1999; Kammerer et al., 1999; Kanemura et al., 1999; Kim et al., 1998). Hence, the expression patterns of these genes raise the speculation as to whether either of them are involved in gliogenesis.

The present study provides several important insights into the function of mammalian Gcm in the nervous system. First, we showed that mouse *Gcm1* and mouse *Gcm2* are expressed in the embryonic brain throughout development by real time PCR. Next, forced expression studies using a retroviral vector indicated that mouse *Gcm1* indeed promotes astrocyte lineage and suppresses neuronal lineage in cultured cells from E12 mouse brains. This induction was so prompt that GFAP$^+$ cells appeared only 2 days after infection. The induction of astrocytes by mouse *Gcm1* was also detected by in utero injection of the retroviral vector into embryonic brains.

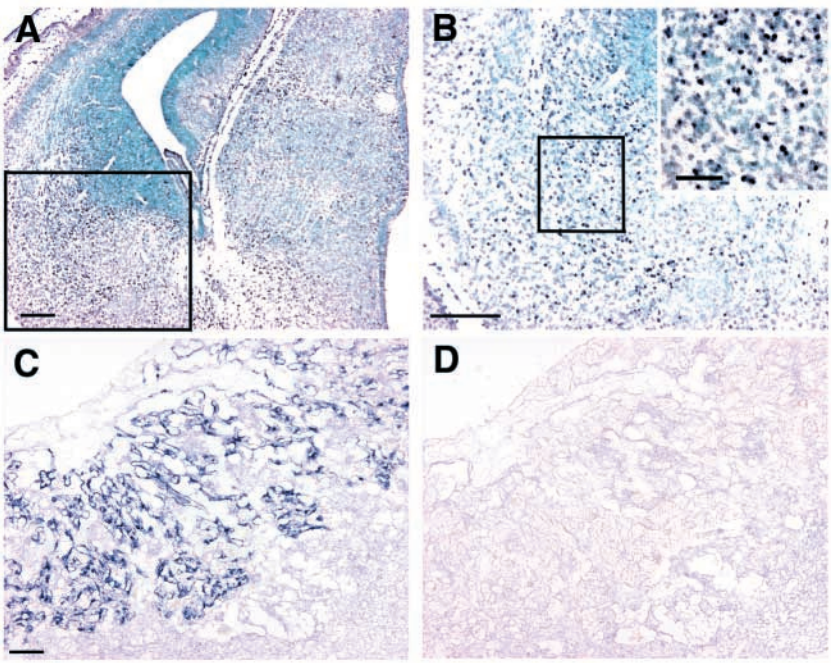

Fig. 6. In situ hybridization analysis of mouse *Gcm1*. Coronal sections of E14.5 mouse forebrains were hybridized with a digoxigenin-labeled mouse *Gcm1* antisense riboprobe (A,B). (B) Higher magnification of the boxed area in A; inset shows higher magnification of the boxed area in B. Dispersed distribution of mouse *Gcm1* mRNA was observed in the ganglionic eminence and in the thalamus. Only a few signals lining the ventricular zone were observed in the cerebral cortex. Sections were counterstained with Methyl Green after in situ hybridization. As controls, sections of E11.5 placenta were hybridized with a digoxigenin-labeled mouse *Gcm1* antisense (C) and sense (D) riboprobes, respectively. mouse *Gcm1* expression was observed only in labyrinth layer of placenta using antisense riboprobe. No signal was observed in embryonic brain sections (not shown), or in placenta sections (D). Scale bars: 150 μm in A-D; 50 μm inset of B.

Previous reports have demonstrated that ectopic expression of mouse *Gcm1* and mouse *Gcm2* in the mouse retina failed to cause neuron-to-glia transformation (Hojo et al., 2000). This discrepancy with our results may be explained by the differences in Müller cells in the retina and in astrocytes referred to in these studies. *Drosophila* has two types of glia, longitudinal and midline glia, but *gcm* is involved only in longitudinal glial differentiation. Mammalian astrocytes exhibit a large heterogeneity differing in morphology, distribution, molecule types expressed, function and cell lineage, including gray matter astrocytes, white matter astrocytes, Müller glia in the retina, Bergman glia in the cerebellum and radial glia. Similar to *Drosophila gcm*, differences in Gcm involvement may occur among these cells.

Our in vivo and in vitro studies have demonstrated that mouse *Gcm1* has the capacity to induce astrocyte lineage cells, but ablation of the mouse *Gcm1* gene did not cause a significant decrease in $GFAP^+$ cells in cultures from mutant brains. Furthermore, while in situ expression of mouse *Gcm1* was detectable in embryonic brains, it did not coincide with the expression of well-known glial lineage markers. These discrepancies strongly suggest that mouse *Gcm1*-expressing cells are a subpopulation of glial cells, distinct from the major astrocyte cell type generated around the P0 in the cortex. Accordingly, data from recent experiments employing retroviral labeling with an ultrasonic injection system have demonstrated a population of early glial lineage existing at E9.5 (McCarthy et al., 2001). This indicates that the specification of some glial cell populations occurs much earlier than believed previously. Our in situ hybridization data revealed many mouse *Gcm1* signals dispersed in the ganglionic eminence and thalamus. The instability of mouse *Gcm1* signals in the brain, however, makes signal detection largely dependent on the conditions of tissue fixation. Our RT-PCR analysis demonstrated that mouse *Gcm1* is expressed at higher levels at E12 than at E14, yet the detectable in situ hybridization signals at E12 were not stronger (data not shown). This might be due to the omission of heart-penetrated perfusion of fixative at E12. Quick fixation may be necessary to avoid degradation of mouse *Gcm1* messages.

Mammalian Gcm exhibits DNA-binding specificity similar to *Drosophila* Gcm (Akiyama et al., 1996; Schreiber et al., 1998). One of the native targets for *Drosophila* Gcm is the *repo* gene, which contains eleven Gcm-binding sites in its upstream region (Akiyama et al., 1996). Gcm-binding sites were also found in trophoblast-specific element 2 (TSE2), which is a cis-element that functions as a placenta-specific enhancer of the human aromatase gene (Yamada et al., 1999). In the present study, we demonstrated that ectopic expression of mouse *Gcm1* in mouse fibroblasts led to the induction of the gene encoding the astrocyte-specific Ca^{2+}-binding protein, S100β. Analysis of the promoter region in the mouse S100β gene revealed the presence of six Gcm-binding-like sequences. Further analysis is necessary to elucidate the regulation of S100β promoter by mouse Gcm1.

It is noteworthy that mouse *Gcm2* and mouse *Gcm1* exhibited similar effects on fibroblasts, but only mouse *Gcm1* induced glial lineage in brain cells. This suggests that mouse Gcm2 is a transcriptional modulator, but is not involved in glial differentiation. However, it has been reported that mouse Gcm2 contains a unique labile domain that suppresses its transcriptional activity by degradation (Tuerk et al., 2000). Thus, it is possible that although mouse Gcm2 has the potential to induce astrocytes, its activities are suppressed under normal circumstances. This raises the speculation that the mouse *Gcm1* defect is compensated by mouse *Gcm2* in our experiments. Further analysis using double knockout mice is necessary to explore this possibility.

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