previous work (Granderath et al., 2000), these results show that both PNTP1 and REPO acts through their respective binding sites in the *loco* promoter to activate transcription.

Synergistic effect of REPO and PNTP1 was also observed in vivo. While ectopic expression of either PNTP1 or REPO in the neuroectoderm caused only a minor increase in the number of cells that expressed a *loco* enhancer-trap strain (rC56), co-expression of REPO and PNTP1 in the neuroectoderm caused a dramatic increase in the response (Fig. 5I-K). When misexpression was directed using the *engrailed*-GAL4 driver, co-expression of REPO and PNTP1 caused a fivefold increase in the number of rC56-expressing cells compared with the expression of REPO or PNTP1 alone (Fig. 5L). Such a synergistic effect was not observed between REPO and TTK69, or between PNTP1 and TTK69 (data not shown). We conclude that the glial expression of *loco* is regulated by the cooperation of REPO and PNTP1.

REPO also suppresses neuronal characteristics

In addition to promoting glial differentiation, the glial determinant GCM also inhibits neuronal differentiation (Hosoya et al., 1995; Jones et al., 1995). This function is probably mediated by glial transcription factors that operate downstream of GCM. Because the results presented above established that REPO cooperates with TTK69 and PNTP1 to direct the expression of glial-specific genes in the CNS, we tested whether these proteins also function to inhibit neuronal differentiation.

Ectopic expression of GCM throughout the neuroectoderm has a profound effect on neuronal differentiation; the number of cells that express the neuron-specific marker ELAV is reduced to 5-15% of that in normal embryos (Hosoya et al., 1995). In stage 13 embryos, ectopic expression of REPO or TTK69 alone caused, respectively, little or a modest reduction in the number of ELAV-positive cells (Fig. 6A,C,D). When these two proteins were co-expressed, however, neuronal differentiation was severely blocked, surpassing the inhibition achieved by the ectopic expression of GCM (Fig. 6B,E). Thus, REPO cooperates with TTK69 not only to promote glial development, but also to inhibit neuronal differentiation.

To address whether *repo* activity is indeed necessary for the inhibition of neuronal differentiation, we took advantage of embryos that ectopically express GCM, in which REPO, TTK69 and PNTP1 are all misexpressed. In these embryos, the number of ELAV-positive cells was greatly reduced, and only short stretches of axons could be recognized by staining with an antibody that labels all CNS axons (Fig. 7B,F). The introduction of a *pointed* mutation into this genotype had little effect on the number of ELAV-positive cells or the axonal phenotype, indicating that *pointed* is not essential for inhibiting neuronal differentiation in this genetic background (Fig. 7D,H). However, removal of *repo* function caused a striking effect on the GCM-misexpression phenotype; ELAV-positive cells increased significantly in number, and they grew long axons that made bundles reminiscent of longitudinal or commissural tracts (Fig. 7C,G). The effect of the *repo* mutation was much more pronounced than the removal of TTK (Fig.

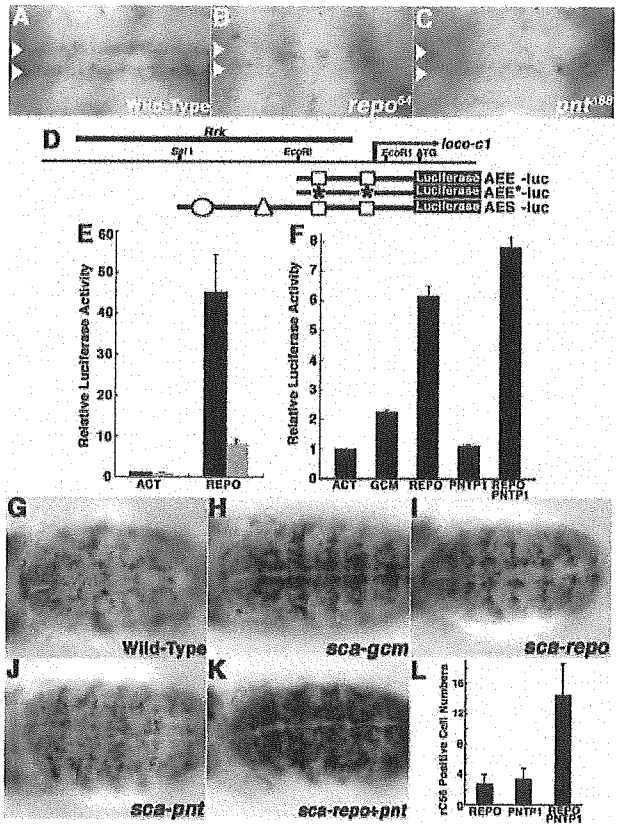

Fig. 5. REPO and PNTP1 cooperate to activate the transcription of *loco*. (A-C) Whole-mount in situ hybridization of stage 16 embryos using the *loco-c1* probe. Ventral views. Wild-type embryos (A) express *loco-c1* mRNA in two rows of longitudinal glial cells (arrowheads). *loco-c1* mRNA expression in longitudinal glia was undetectable in *repo* mutant embryos (B) or in *pointed* mutant embryos (C). Anterior is leftwards. (D) Structure of the *loco-c1* promoter-luciferase reporter genes used for transfection assays. AEE-*luc* carries a 0.7 kb *loco* promoter fragment, which contain two CAATTA motifs (square). Single base changes (asterisk) were introduced in both motifs in AEE*-*luc* reporter. AES-*luc* has a 1.4 kb *loco* fragment, which includes an Ets-binding site (triangle) and a GCM-binding site (oval), both identified by Granderath et al. (Granderath et al., 2000). The glial enhancer fragment (Rrk) used by Granderath et al. (Granderath et al., 2000) is shown above the map. The exact position of the transcriptional start site is not known. (E) REPO activates transcription through the CAATTA motif in the *loco-c1* promoter. S2 cells were transfected with AEE-*luc* (black) or AEE*-*luc* (gray) reporter and REPO-expressing plasmid (REPO) or the empty vector (ACT). Luciferase activity obtained after the transfection of the effector constructs was normalized to the activity of the AEE-*luc* reporter co-transfected with the empty vector. (F) REPO and PNTP1 cooperates on the expression of the *loco* promoter. S2 cells were co-transfected with the AES-*luc* reporter gene and effector constructs that expressed GCM (column 2), REPO (column 3), PNTP1 (column 4) or REPO and PNTP1 (column 5). Luciferase activity was normalized to the value obtained with the empty vector (column 1). (G-L) REPO and PNTP1 has synergistic effects on *loco* expression. (G-K) *lacZ* expression of rC56, an enhancer trap insertion into the *loco* locus. The following transgenes were misexpressed in the entire neuroectoderm using the *scabrous*-GAL4 strain: (G) none; (H) GCM; (I) REPO; (J) PNTP1; (K) REPO and PNTP1. (L) Number of rC56-positive cells upon misexpression using the *engrailed*-GAL4 driver. The number of rC56-positive cells in the *engrailed*-positive region in each segment were scored (n=15).

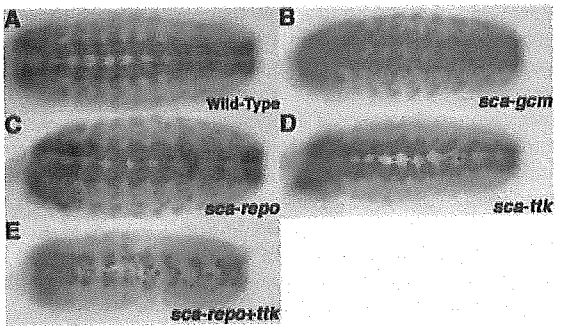

Fig. 6. REPO and TTK69 cooperate to suppress neuronal development. The expression of the neuron-specific protein ELAV in stage 13 embryos. (A) Wild type. (B-E) Ectopic expression using the *scabrous* GAL4 driver. (B) Ectopic expression of GCM reduced the number of ELAV-expressing cells. Ectopic expression of REPO (C) or TTK69 (D) caused, respectively, little or a modest reduction in the number of ELAV-expressing cells. When REPO and TTK69 were co-expressed, they synergistically reduced the number of ELAV-positive cells (E). Anterior is leftwards.

7I,J), which has been regarded as the major factor in repressing neuronal differentiation in glial cells. As the expression of the glial marker M84 was still absent (Fig. 7C), the rescue of neuronal differentiation was achieved without normal glial function, most probably through an autonomous effect on presumptive neurons. We conclude that REPO is an essential factor for mediating the function of GCM, both to promote glial development and to inhibit neuronal differentiation.

DISCUSSION

REPO is the major factor for glial differentiation

Although glial specification by GCM is well established, how the characteristics of individual glial cells are determined is poorly understood. GCM expression is confined to the early stage of glial development, suggesting that GCM itself does not participate in the terminal differentiation of glia. Moreover, GCM also directs blood cell development; GCM is expressed in macrophage precursors and ectopic expression of GCM in crystal cell precursors causes the transformation of crystal cells to macrophages (Bernardoni et al., 1997; Lebestky et al., 2000). These results clearly show that the expression of GCM does not always lead to the determination and terminal differentiation of glia. In glial cells, GCM induces the expression of three transcription factors, REPO, TTK69, and PNTP1 (Hosoya et al., 1995; Jones et al., 1995; Giesen et al., 1997), and the loss of these proteins causes abnormal glial development, although GCM expression remains normal (Halter et al., 1995; Giesen et al., 1997) (data not shown). Although *gcm* can direct *repo* expression in various contexts (Akiyama-Oda et al., 1998; Bernardoni et al., 1998), *repo* is not expressed endogenously in blood cells, but is confined to GCM-positive glial cells, lasting even after *gcm* expression has ceased. In *repo* mutant embryos, the migration, survival and terminal differentiation of glial cells are abnormal (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995). In this paper, we showed that the homeodomain protein REPO activates gene expression in glia, and also demonstrated that REPO mediates the suppression of neuronal differentiation (Fig. 8). These results suggest that REPO is the major factor that is necessary for glial development.

Target specificity of REPO is controlled by a combination of cooperating factors

Identifying the target genes of homeodomain proteins has been difficult, partly because most homeodomain proteins bind similar DNA sequences in vitro. REPO has been shown to bind the CAATTA motif in vitro (Halter et al., 1995), and we showed that REPO is necessary and sufficient to activate the transcription of the *ftz* HDS reporter gene that carries the CAATTA motif in its promoter. In cultured *Drosophila* cells, the ability of REPO to activate the CAATTA-*luc* reporter gene was dependent on the presence of the REPO homeodomain and the CAATTA motif in the reporter gene.

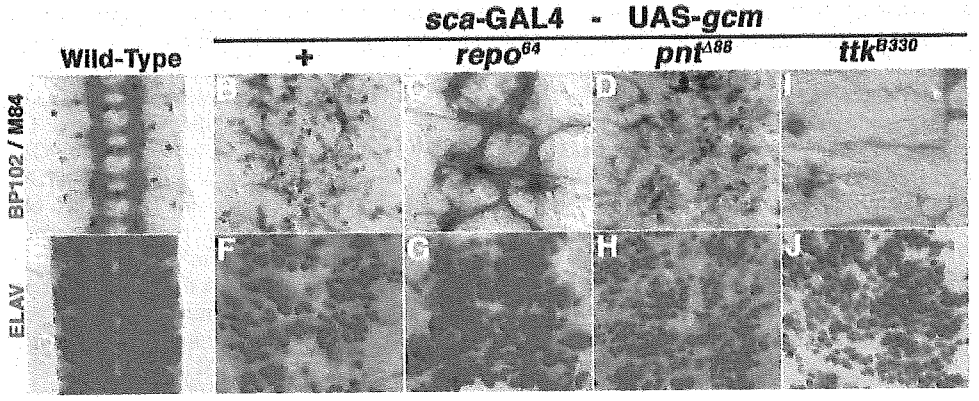

Fig. 7. REPO is necessary for the inhibition of neuronal differentiation by GCM. (A-D,I) The axonal scaffold was labeled using mAb BP102 (brown). (A-D) Glial cells were labeled by β-galactosidase expression from the glial marker M84 (black). (E-H,J) All neurons in the CNS were labeled using the ELAV antibody. (A,E) Wild type. (B,F) Ectopic expression of GCM expression using the *scabrous GAL4* driver. Ectopic expression of GCM in the *repo* mutant background (C,G), *pointed* mutant background (D,H) or *ttk* mutant background (I,J). Ectopic expression of GCM caused a reduction in the number of ELAV-positive neurons and axonal extension, as well as an increased number of cells that expressed the M84 marker (B,F). Removal of *repo* function resulted in a dramatic restoration of ELAV-positive cells and axonal development (C,G). The effect of removing *pointed* (D,H) or *ttk* (I,J) function was, respectively, undetectable or minor (compare with B,F). Note that the *ttk* mutant (I,J) is labeled for axons (I; mAB BP102) and neurons (J; ELAV); this animal did not carry the glial marker M84 (I,J). All embryos were stage 15. Anterior is upwards.

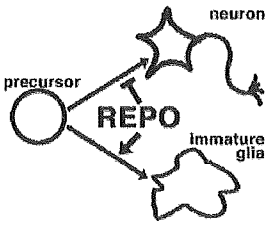

Fig. 8. Model of glial development. Glial cell development involves two processes that both depend on REPO: the activation of glial differentiation and the inhibition of neuronal differentiation.

These results strongly suggest that REPO activates the transcription of the *ftz* HDS reporter gene by directly binding to the CAATTA motif in vivo.

Despite the glia-specific expression of the *ftz* HDS reporter gene, the CAATTA motif is not a specific target site of REPO. FTZ and EN bind the CAATTA motif (Desplan et al., 1988; Schier and Gehring, 1992), which also resembles the consensus binding sequence for ANTP and UBX (Müller et al., 1988; Ekker et al., 1992). Why do other homeodomain proteins fail to drive the *ftz* HDS reporter gene in vivo? Recent results show that homeodomain proteins require co-factors to activate the transcription of their target genes. Co-factors, such as EXD and FTZF1, are also DNA-binding proteins that require specific binding sites in the target gene (Chan et al., 1994; van Dijk and Murre, 1994; Guichet et al., 1997; Yu et al., 1997). Homeodomain proteins other than REPO may be incapable of activating the *ftz* HDS reporter gene because it does not have binding sites for their co-factors.

The behavior of the *ftz* HDS reporter gene suggests that the requirement for co-factors may also apply to REPO. Although the ectopic expression of REPO induced the ectopic expression of the *ftz* HDS reporter gene in the periphery, it did not affect the expression pattern in the CNS. Thus, REPO cannot be the single factor responsible for the activation of the *ftz* HDS reporter gene. Indeed, the expression pattern of the *ftz* HDS reporter gene is altered by changing nucleotides outside the CAATTA motif (Nelson and Laughon, 1993), indicating that *ftz* HDS contains binding sites for factors other than REPO. The simplest interpretation is that such factors are present in the periphery, but not in the CNS.

Using additional target genes of REPO, we provided further evidence that the transcriptional regulation by REPO involves co-factors. Although the enhancer-trap line M84 and the *loco* gene were both dependent on REPO function, and could be expressed precociously and ectopically upon mis-expression of REPO, much stronger responses were obtained when REPO was co-expressed with TTK69 or PNTP. Endogenous expression of M84 and the *loco* gene occurs in cells that co-express REPO and TTK69 or PNTP1, respectively. TTK69 and PNTP1 are thus good candidates for REPO co-factors. Together with an earlier study (Granderath et al., 2000) our results show that REPO and PNTP1 cooperate on *loco* expression through their binding sites in the *loco* promoter. Likewise the synergism between REPO and TTK69 may also occur on the promoter of their target genes.

Our conclusion that the expression of M84 and *loco* are achieved by a cooperation of REPO and TTK69/PNTP1 does not rule out the possibility that these genes are also direct targets of GCM. In fact, reporter genes driven by *loco* enhancer elements are expressed normally in stage 14 *repo* mutant embryos, indicating that other factor(s) activate their transcription at the onset of gliogenesis (Campbell et al., 1994; Granderath et al., 2000). Because the *loco* enhancer element contains GCM-binding sites, GCM can directly regulate *loco* (Granderath et al., 2000). However, as the expression of GCM in glia is transient, transcription initiated by GCM must be sustained by other factors. REPO and PNTP1 are the best candidates for factors that maintain *loco* expression throughout glial development and functioning.

The synergistic effect of REPO and TTK69 on M84 marker expression suggests a positive role of TTK69 on glial differentiation. As the major function of TTK69 has been thought to be the inhibition of neuronal differentiation through transcriptional repression (Brown et al., 1991; Read et al., 1992; Xiong and Montell, 1993; Giesen et al., 1997), the positive action of TTK69 on glial gene expression could be an indirect effect through repressing transcription of a repressor for M84 expression. However, TTK69 can activate transcription in yeast cells (Yu et al., 1999), suggesting that TTK69 may also promote transcription, depending on the cellular context. Recent studies also implicate a role of TTK69 in cell proliferation, through controlling the expression of regulators of the cell cycle (Badenhorst, 2001; Baonza et al., 2002). Badenhorst (Badenhorst, 2001) has shown that overexpression of TTK69 results in the inhibition of glial development, accompanied by the repression of the S-phase cyclin and glial proliferation. As we observe an increase in the number of cells that express M84 glial marker upon co-expression of TTK69 and REPO, our result cannot be accounted for by the ability of TTK69 to inhibit glial cell cycle. Whereas ectopic expression of TTK69 reduces the expression of the endogenous *repo* gene (Badenhorst, 2001), our misexpression paradigm provides exogenous REPO through the GAL4/UAS control. Thus the existence of REPO might modify the activity of TTK69, so that it plays a positive role on glial development.

Repression of neuronal differentiation during glial development

Glial fate determination involves not only the promotion of glial differentiation but also the suppression of neuronal properties. Because ectopic GCM can induce neurogenesis in certain contexts (Akiyama-Oda et al., 1998; Van de Bor et al., 2002), it is unlikely that GCM directly represses neuronal differentiation. TTK69 has been proposed to inhibit neuronal differentiation, mainly because of its loss-of-function phenotype in the sensory organ (Giesen et al., 1997). Here, we have shown that the co-expression of REPO and TTK69 has a potent neuron-suppressing activity, and further demonstrated that the *repo* mutant permits neuronal differentiation even when GCM is overexpressed. This strongly suggests that REPO functions not only to activate the transcription of glial genes, but also to prevent the neuronal differentiation of presumptive glial cells (Fig. 8).

If glia and neuron represent two mutually exclusive cell states that must be chosen between early in development, it is somewhat strange that suppression of neuronal development should be carried out by proteins that are expressed throughout

glial differentiation. The existence of continuous suppression of neuronal properties in glia suggests that cells within the nervous system may retain the potential to become neurons or glia throughout their cellular history. This idea is supported by the observation that GCM is able to transform post-mitotic neurons into glia (Jones et al., 1995). Conversely, in the vertebrate nervous system, glial cells (astrocytes and oligodendrocyte-precursor) can respond to environmental signals and function as neural stem cells, generating neurons (Doetsch et al., 1999; Kondo and Raff, 2000). The role of REPO and TTK69 may be to suppress the ability of glia to respond to cues that would cause them to change into neurons or neural precursors.

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REFERENCES

Akiyama, Y., Hosoya, T., Poole, A. M. and Hotta, Y. (1996). The gcm-motif: a novel DNA-binding motif conserved in *Drosophila* and mammals. *Proc. Natl. Acad. Sci. USA* **93**, 14912-14916.

Akiyama-Oda, Y., Hosoya, T. and Hotta, Y. (1998). Alteration of cell fate by ectopic expression of *Drosophila glial cells missing* in non-neural cells. *Dev. Genes Evol.* **208**, 578-585.

Albagli, O., Klaes, A., Ferreira, E., Leprince, D. and Klämbt, C. (1996). Function of *ets* genes is conserved between vertebrates and *Drosophila*. *Mech. Dev.* **59**, 29-40.

Badenhorst, P. (2001). Tramtrack controls glial number and identity in the *Drosophila* embryonic CNS. *Development* **128**, 4093-4101.

Baonza, A., Murawsky, C. M., Travers, A. A. and Freeman, M. (2002). Pointed and Tramtrack69 establish an EGFR-dependent transcriptional switch to regulate mitosis. *Nat. Cell Biol.* **4**, 976-980.

Bernardoni, R., Vivancos, V. and Giangrande, A. (1997). *glide/gcm* is expressed and required in the scavenger cell lineage. *Dev. Biol.* **191**, 118-130.

Bernardoni, R., Miller, A. A. and Giangrande, A. (1998). Glial differentiation does not require a neural ground state. *Development* **125**, 3189-3200.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brown, J. L., Sonoda, S., Ueda, H., Scott, M. P. and Wu, C. (1991). Repression of the *Drosophila fushi tarazu* (*ftz*) segmentation gene. *EMBO J.* **10**, 665-674.

Brown, J. L. and Wu, C. (1993). Repression of *Drosophila* pair-rule segmentation genes by ectopic expression of *tramtrack*. *Development* **117**, 45-58.

Campbell, G., Göring, H., Lin, T., Spana, E., Andersson, S., Doe, C. Q. and Tomlinson, A. (1994). RK2, a glial-specific homeodomain protein required for embryonic nerve cord condensation and viability in *Drosophila*. *Development* **120**, 2957-2966.

Campos-Ortega, J. A. and Hartenstein, V. (1997). Stages of *Drosophila* embryogenesis. In *The Embryonic Development of* Drosophila melanogaster, pp. 9-102. Berlin: Springer.

Chan, S. K., Jaffe, L., Capovilla, M., Botas, J. and Mann, R. S. (1994). The DNA binding specificity of Ultrabithorax is modulated by cooperative interactions with extradenticle, another homeoprotein. *Cell* **78**, 603-615.

Desplan, C., Theis, J. and O'Farrell, P. H. (1988). The sequence specificity of homeodomain-DNA interaction. *Cell* **54**, 1081-1090.

Doetsch, F., Caillé, I., Lim, D. A., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97**, 703-716.

Ekker, S. C., von Kessler, D. P. and Beachy, P. A. (1992). Differential DNA sequence recognition is a determinant of specificity in homeotic gene action. *EMBO J.* **11**, 4059-4072.

Gabay, L., Scholz, H., Golembo, M., Klaes, A., Shilo, B. Z. and Klämbt, C. (1996). EGF receptor signaling induces *pointed P1* transcription and inactivates Yan protein in the *Drosophila* embryonic ventral ectoderm. *Development* **122**, 3355-3362.

Giesen, K., Hummel, T., Stollewerk, A., Harrison, S., Travers, A. and Klämbt, C. (1997). Glial development in the *Drosophila* CNS requires concomitant activation of glial and repression of neuronal differentiation genes. *Development* **124**, 2307-2316.

Granderath, S., Stollewerk, A., Greig, S., Goodman, C. S., O'Kane, C. J. and Klämbt, C. (1999). *loco* encodes an RGS protein required for *Drosophila* glial differentiation. *Development* **126**, 1781-1791.

Granderath, S., Bunse, I. and Klämbt, C. (2000). gcm and pointed synergistically control glial transcription of the *Drosophila* gene *loco*. *Mech. Dev.* **91**, 197-208.

Guichet, A., Copeland, J. W. R., Erdélyi, M., Hlousek, D., Závorszky, P., Ho, J., Brown, S., Percival-Smith, A., Krause, H. M. and Ephrussi, A. (1997). The nuclear receptor homologue Ftz-F1 and the homeodomain protein Ftz are mutually dependent cofactors. *Nature* **385**, 548-552.

Halter, D. A., Urban, J., Rickert, C., Ner, S. S., Ito, K., Travers, A. A. and Technau, G. M. (1995). The homeobox gene *repo* is required for the differentiation and maintenance of glia function in the embryonic nervous system of *Drosophila melanogaster*. *Development* **121**, 317-332.

Harrison, S. D. and Travers, A. A. (1990). The *tramtrack* gene encodes a *Drosophila* finger protein that interacts with the *ftz* transcriptional regulatory region and shows a novel embryonic expression pattern. *EMBO J.* **9**, 207-216.

Hosoya, T., Takizawa, K., Nitta, K. and Hotta, Y. (1995). *glial cells missing*: a binary switch between neuronal and glial determination in Drosophila. *Cell* **82**, 1025-1036.

Ito, K., Urban, J. and Technau, G. M. (1995). Distribution, classification, and development of *Drosophila* glial cells in the late embryonic and early larval ventral nerve cord. *Roux's Arch. Dev. Biol.* **204**, 284-307.

Jones, B. W., Fetter, R. D., Tear, G. and Goodman, C. S. (1995). *glial cells missing*: a genetic switch that controls glial versus neuronal fate. *Cell* **82**, 1013-1023.

Klaes, A., Menne, T., Stollewerk, A., Scholz, H. and Klämbt, C. (1994). The Ets transcription factors encoded by the Drosophila gene *pointed* direct glial cell differentiation in the embryonic CNS. *Cell* **78**, 149-160.

Klämbt, C. (1993). The *Drosophila* gene *pointed* encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development* **117**, 163-176.

Klämbt, C. and Goodman, C. S. (1991). The diversity and pattern of glia during axon pathway formation in the Drosophila embryo. *Glia* **4**, 205-213.

Klämbt, C., Jacobs, J. R. and Goodman, C. S. (1991). The midline of the Drosophila central nervous system: a model for the genetic analysis of cell fate, cell migration, and growth cone guidance. *Cell* **64**, 801-815.

Kondo, T. and Raff, M. (2000). Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. *Science* **289**, 1754-1757.

Kramer, S., West, S. R. and Hiromi, Y. (1995). Cell fate control in the *Drosophila* retina by the orphan receptor seven- up: its role in the decisions mediated by the ras signaling pathway. *Development* **121**, 1361-1372.

Krasnow, M. A., Saffman, E. E., Kornfeld, K. and Hogness, D. S. (1989). Transcriptional activation and repression by *Ultrabithorax* proteins in cultured Drosophila cells. *Cell* **57**, 1031-1043.

Lai, Z. C. and Li, Y. (1999). Tramtrack69 is positively and autonomously required for Drosophila photoreceptor development. *Genetics* **152**, 299-305.

Lai, Z. C., Harrison, S. D., Karim, F., Li, Y. and Rubin, G. M. (1996). Loss of tramtrack gene activity results in ectopic R7 cell formation, even in a *sina* mutant background. *Proc. Natl. Acad. Sci. USA* **93**, 5025-5030.

Lebestky, T., Chang, T., Hartenstein, V. and Banerjee, U. (2000). Specification of *Drosophila* hematopoietic lineage by conserved transcription factors. *Science* **288**, 146-149.

Lehmann, R. and Tautz, D. (1994). *In situ* hybridization to RNA. In *Methods in Cell Biology*. Vol. 44 (ed. Goldstein and Fyrberg), pp. 575-598. San Diego: Academic press.

Li, S., Li, Y., Carthew, R. W. and Lai, Z. C. (1997). Photoreceptor cell differentiation requires regulated proteolysis of the transcriptional repressor Tramtrack. *Cell* **90**, 469-478.

Mayer, U. and Nüsslein-Volhard, C. (1988). A group of genes required for pattern formation in the ventral ectoderm of the *Drosophila* embryo. *Genes Dev.* **2**, 1496-1511.

Müller, M., Affolter, M., Leupin, W., Otting, G., Wüthrich, K. and Gehring, W. J. (1988). Isolation and sequence-specific DNA binding of the *Antennapedia* homeodomain. *EMBO J.* **7**, 4299-4304.

Nelson, H. B. and Laughon, A. (1993). *Drosophila* glial architecture and development analysis using a collection of new cell-specific markers. *Roux's Arch. Dev. Biol.* **202**, 341-354.

O'Neill, E. M., Rebay, I., Tjian, R. and Rubin, G. M. (1994). The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. *Cell* **78**, 137-147.

Patel, N. H. (1994). Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. In *Methods in Cell Biology*, vol. 44 (ed. Goldstein and Fyrberg), pp. 445-487. San Diego: Academic Press.

Read, D., Levine, M. and Manley, J. L. (1992). Ectopic expression of the *Drosophila tramtrack* gene results in multiple embryonic defects, including repression of *even-skipped* and *fushi tarazu*. *Mech. Dev.* **38**, 183-195.

Read, D. and Manley, J. L. (1992). Alternatively spliced transcripts of the *Drosophila tramtrack* gene encode zinc finger proteins with distinct DNA binding specificities. *EMBO J.* **11**, 1035-1044.

Salzberg, A., D'Evelyn, D., Schulze, K. L., Lee, J. K., Strumpf, D., Tsai, L. and Bellen, H. J. (1994). Mutations affecting the pattern of the PNS in Drosophila reveal novel aspects of neuronal development. *Neuron* **13**, 269-287.

Schier, A. F. and Gehring, W. J. (1992). Direct homeodomain-DNA interaction in the autoregulation of the *fushi tarazu* gene. *Nature* **356**, 804-807.

Van de Bor, V., Heitzler, P., Leger, S., Plessy, C. and Giangrande, A. (2002). Precocious expression of the Glide/Gcm glial-promoting factor in Drosophila induces neurogenesis. *Genetics* **160**, 1095-1106.

van Dijk, M. A. and Murre, C. (1994). *extradenticle* raises the DNA binding specificity of homeotic selector gene products. *Cell* **78**, 617-624.

Vincent, S., Vonesch, J. L. and Giangrande, A. (1996). *glide* directs glial fate commitment and cell fate switch between neurones and glia. *Development* **122**, 131-139.

Xiong, W. C. and Montell, C. (1993). *tramtrack* is a transcriptional repressor required for cell fate determination in the *Drosophila* eye. *Genes Dev.* **7**, 1085-1096.

Xiong, W. C., Okano, H., Patel, N. H., Blendy, J. A. and Montell, C. (1994). *repo* encodes a glial-specific homeo domain protein required in the *Drosophila* nervous system. *Genes Dev.* **8**, 981-994.

Yu, Y., Li, W., Su, K., Yussa, M., Han, W., Perrimon, N. and Pick, L. (1997). The nuclear hormone receptor Ftz-F1 is a cofactor for the *Drosophila* homeodomain protein Ftz. *Nature* **385**, 552-555.

Yu, Y., Yussa, M., Song, J., Hirsch, J. and Pick, L. (1999). A double interaction screen identifies positive and negative *ftz* gene regulators and ftz-interacting proteins. *Mech. Dev.* **83**, 95-105.

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Grafted Swine Neuroepithelial Stem Cells Can Form Myelinated Axons and Both Efferent and Afferent Synapses With Xenogeneic Rat Neurons

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Neuroepithelial stem cells derived from the swine mesencephalic neural tube were examined regarding their eligibility for neural xenografting as a donor material, with the aim of evaluating myelinated axon formation and both types of synaptic formation with xenogeneic host neurons as part of possible neural circuit reconstruction. The mesencephalic neural tube tissues were dissected out from swine embryos at embryonic days 17 and 18 and were implanted immediately into the striatum of the Parkinsonian model rat. The swine-derived grafts had many nestin-positive rosette-forming, neurofilament-positive, and tyrosine hydroxylase-positive cells in the rat striatum. Electron microscopic study revealed both efferent and afferent synaptic formations in the donor-derived immature neurons or tyrosine hydroxylase-positive donor cells in the grafts. Myelinated axons, both positive and negative for swine-specific neurofilament antibody, were mingled together in the graft. These results indicated that implanted neuroepithelial stem cells could survive well and divide asymmetrically into both nestin-expressing precursors and differentiated neurochemical marker-expressing neurons in the xenogeneic rat striatum, with the help of an immunosuppressant. Donor-derived immature neurons formed both efferent and afferent synapses with xenogeneic host neurons, and donor-derived axons were myelinated, which suggests that implanted swine neuroepithelial stem cells could possibly restore damaged neuronal circuitry in the diseased brain.

Key words: swine; neuroepithelial stem cell; neural xenograft; myelin; synapse

Reynolds and Weiss (1992) successfully expanded stem cells from the adult brain in vitro, and over the past decade much attention has been focused on the neural stem cell and its multipotency and self-renewing characteristics as a potential donor material for neural grafting (Fisher, 1997; Stemple and Mahanthappa, 1997). Because of the self-renewal characteristic of the stem cells, they naturally remain into adulthood in situ. However, their features change with age, and we propose that younger stem cells such as neuroepithelial stem cells (NESCs) might have greater potential in repairing damaged neural circuitry than older ones, because younger stem cells have a greater potential for differentiating into neurons (Uchida et al., 1995, 1999; Qian et al., 2000). A recent report indicated, on the other hand, that neural stem cells from the adult brain tended to differentiate more into astrocytes than into neurons (Gritti et al., 1996; Palmer et al., 1999). Reconstruction of damaged neuronal circuitry can best be achieved by grafting of cells with a vigorous neuronal differentiation capability, and this property is unique to NESCs placed in the target of interest by transplantation techniques. Additional desirable capabilities include vigorous axonal growth and migratory potential to allow diffuse interaction between the grafted tissue and the host brain, which has been shown previously in rat and mouse NESCs both in vitro and in vivo (Uchida et al., 1995; Hara et al., 2000). Transplantation therapy can be used to achieve a number of goals, for example, replacement or augmentation of deficient substances (Uchida et al., 1992). However, in the technique proposed here, the main or even sole aim of our transplantation therapy is the reconstruction of damaged neuronal circuits using xenogeneic stem cell-derived neurons.

The most obvious candidate would be the NESCs from a human fetus, but the ethical and practical problems rule out the use of human fetal tissue in such applications. A valid alternative must therefore be sought. In many

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ways, the pig brain resembles the human brain (Larsson et al., 2001). The current study was designed, using the rat model, to assess the feasibility of using stem cells from the mesencephalic neural tube of the early-stage pig fetus. This study examined both the ability of the transplanted swine cells to survive in the xenogeneic pathological environment and the capability of the transplanted stem cells to differentiate into neurons. Furthermore, we focused on whether the stem cells can form both efferent and afferent synapses with the xenogeneic host neurons and whether myelination of the donor-derived axons occurs.

MATERIALS AND METHODS

All experimental animals were handled and all animal experiments were performed according to guidelines of the Animal Experimental Committee of Keio University and Central Institute for Experimental Animals.

Viability of the Donor Cells

Before proceeding with the harvest and transplantation of the porcine stem cells, the presence of viable stem cells in the donor material was verified. Before embryonic day 16 (E16), it was practically difficult to identify the fetus in the uterus. Therefore, E17–18 Yorkshire swine fetuses (Fujieda Farm, Shizuoka, Japan) were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 16 hr at room temperature and embedded in paraffin following treatment with ethanol and xylene. The 4-μm-thick transverse sections were taken through the mesencephalic region of E17 or E18 pig fetuses, which were similar to those used for the actual donor material, and sequentially incubated with the following: 1) 5% goat serum in PBS for 60 min at room temperature, 2) nestin (rabbit polyclonal IgG; 1:500; H.O., unpublished) and neuron-specific βIII-tubulin (mouse monoclonal IgG; 1:500; Tuj-1; Covance) antisera in PBS containing 1% bovine serum albumin (BSA) for 16 hr at 4°C, and 3) Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and Alexa Fluor 568 goat anti-mouse IgG (Molecular Probes) diluted 1:500 in PBS containing 1% BSA. Musashi-1 staining was carried out according to the method described previously (Kaneko et al., 2000; Keyoung et al., 2001) using the anti-Musashi-1 antibody (rat monoclonal IgG; clone 14H1) diluted 1:500.

For electron microscopy (EM), E17–18 pig fetuses were fixed with 2% paraformardehyde and 1% glutaraldehyde in PBS. The mesencephalic neural tube region was carefully excised and postfixed with 1% osmium tetroxide for 1 hr and dried in a graded series of ethanol concentrations (50%, 70%, 90%, 95%, and 100%) followed by acetone and QY-1 (acetone:n-butylglycidyl ether = 1:1; Oken Shoji, Tokyo, Japan), then embedded in Epok 812 (Oken Shoji). Ultrathin sections were made, stained with lead citrate, and examined by EM.

Stem Cell Harvesting

Five- to six-month-old pregnant Yorkshire sows (150–250 kg; Fujieda Farm) at gestational day 17 or 18 were anesthetized with azaperone (2 mg/kg, i.m.), ketamine (5 mg/kg, i.m.), and pentobarbiturate (10 mg/kg, i.v.), and the fetuses (approximately 10 per animal) were delivered by cesarean section. Immediately after delivery, the fetuses were pretreated with an enzyme cocktail consisting of 0.5% trypsin and 0.25% pancreatin for 15 min at 4°C to facilitate complete removal of the mesenchyme from the neural tube. The mesencephalic neural tube was exposed, and a section of the neural tube was dissected out under the dissecting microscope. The section was approximately 100-μm of membranous tissue in the shape of a butterfly. The dissected tissue was placed in a Dulbecco's modified Eagle's medium bubble and was aspirated using a Hamilton syringe. This was repeated twice more, so that there were three pieces of tissue in the syringe.

Preparation of the Host and Transplantation

Four-week-old male Wistar rats (Experimental Animals Supply Co., Ltd., Saitama, Japan) were used to create a hemi-Parkinsonian rat model by a previously described method (Studer et al., 1998). In total, 31 Parkinsonian model rats that met the damage criteria using methamphetamine (3 mg/kg, i.p.; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) were deeply anesthetized and placed on a stereotactic frame (Narishige Scientific Instrument Lab., Tokyo, Japan). Three pieces of freshly excised pig fetal neural tube, prepared as described above, were injected into the right striatum of each rat. The coordinates were 3 mm lateral from the midline at the bregma level and a depth of 5 mm from the surface of the brain, with the incisor bar fixed at 3.3 mm below the interaural line. FK506 (donated by Fujisawa Pharmaceutical Co., Osaka, Japan) at 1 mg/kg/day and cyclophosphamide (donated by Shionogi & Co., Ltd., Osaka, Japan) at 10 mg/kg/day were given i.m. and i.p., respectively, on days 0–7 of transplantation (Murase et al., 1993).

Histology and Immunohistochemistry

Four to eight weeks posttransplantation, rats were sacrificed by an overdose of pentobarbiturate and perfused through the heart with physiological saline, followed by 4% paraformaldehyde in PBS. Specimens were prepared for both paraffin and cryostat sectioning. The paraffin-embedded sections were processed as described above and stained for nestin expression. After deparaffinization, the 4-μm-thick coronal sections were incubated with the following: 1) 0.3% H_2O_2 in methanol for 20 min at room temperature, 2) 5% goat serum in PBS for 60 min at room temperature, 3) antinestin antibody (1:500) in PBS containing 1% BSA for 20 hr at 4°C, 4) biotinylated anti-rabbit antibody for 40 min at room temperature, 5) avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC Reagent; Vector Laboratories, Burlingame, CA) for 60 min at room temperature, and 6) Tris-buffered saline (TBS) containing 0.2 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.003% H_2O_2. Finally, specimens were counterstained with hematoxylin.

For cryostat sectioning, the brains were removed and kept in the 4% paraformardehyde overnight at 4°C. The brains were then treated with 10% sucrose in PBS, followed by 15%, 20%, and 30% sucrose, and then were embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co., Ltd., Tokyo Japan) and frozen in acetone and dry ice. Subsequently, the brains were cryosectioned coronally with a cryostat in 10-μm-thick slices and mounted on slides coated with poly-L-lysine. Some of the slides were routinely stained with hematoxylin-eosin (H-E). The remainder of the slides proceeded to immunohistochemical

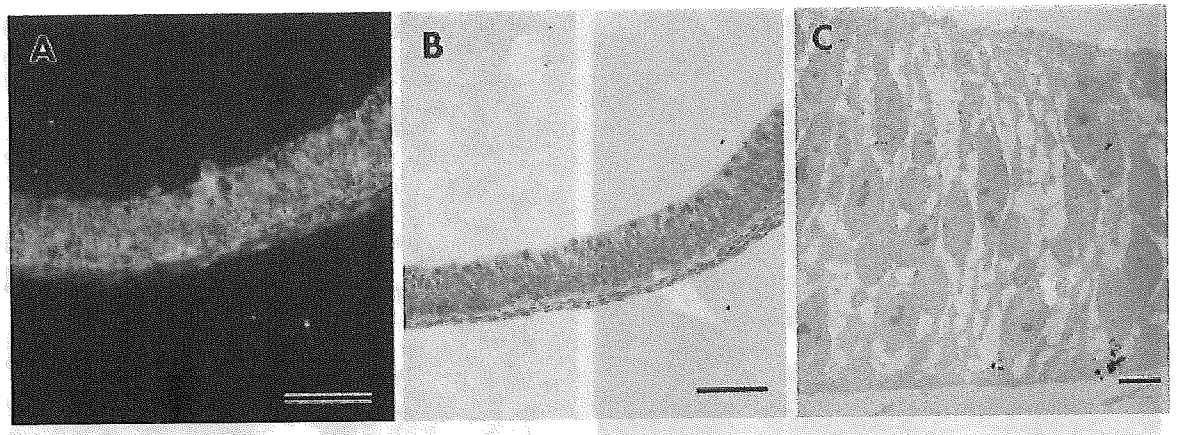

Fig. 1. Immunohistochemical and EM findings in E18 porcine donor tissue. **A:** Double staining for nestin and Tuj-1 reveals nestin-positive cells (green) distributed diffusely throughout the mesencephalic neural part of the E18 swine fetus, with Tuj-1-positive cells (red) seen in the extreme lateral portion of the neural tube. **B:** With some cells in the neural tube staining strongly positively and others less strongly positively, all cells in the neural tube stain positively for stem cell marker Musashi-1 (brown), with remarkably large nuclei counterstained by hematoxylin. **C:** EM findings for the neural tube at this stage reveal typical immature, pseudostratified cellular morphology, with an absence of organelles and a large N/C ratio. Scale bars = 100 μm in A,B; 10 μm in C.

analysis for neurofilament and tyrosine hydroxylase (TH) expression. Specimens were washed three times with PBS and then sequentially incubated with the following for swine-neurofilament staining: 1) 5% rabbit serum in PBS for 60 min at room temperature, 2) neurofilament antiserum (mouse monoclonal IgG; 1:100; Dako, Glostrup, Denmark) in PBS containing 1% BSA for 16 hr at 4°C, 3) horseradish peroxidase-conjugated anti-mouse IgG (BioMakor, Rehovot, Israel) diluted 1:200 for 40 min at room temperature, and 4) TBS containing 0.2 mg/ml DAB and 0.003% H_2O_2.

For TH immunostaining, specimens were incubated with the following: 1) 5% goat serum in PBS for 60 min at room temperature, 2) TH antiserum (rabbit polyclonal IgG; 1:500; Genosys Biotechnologies Inc., Cambridge, United Kingdom) in PBS containing 1% BSA for 16 hr at 4°C, 3) horseradish peroxidase-conjugated anti-rabbit IgG (BioMakor) diluted 1:400 for 40 min at room temperature, and 4) TBS containing 0.2 mg/ml DAB and 0.003% H_2O_2.

The neurofilament antibody used was specific for pig, among other species. The manufacturer's instructions had no information on its use in the rat, but we confirmed that it reacted negatively with rat brain tissue through Western blotting analysis (data not shown). The TH antibody used was confirmed as reacting positively with pig tissue by Western blotting analysis using a pig adrenal medulla and pig midbrain (data not shown) and according to the manufacturer's instructions was positive also for rat tissue, which we also confirmed.

EM and Immuno-EM for the Grafts

Rats were sacrificed by an overdose of pentobarbiturate and perfused through the heart with physiological saline, followed by 2% paraformardehyde and 1% glutaraldehyde in PBS. Grafted areas were carefully excised and then treated with the same method as described for EM for pig fetuses.

For immuno-EM, grafted areas of brain were removed and cut into 2-mm-thick slices on a vibratome. The specimens were then washed in a graded series of sucrose concentrations (10%, 15%, 20%, 25%, and 25% sucrose + 5% glyceol) in PBS. Frozen sections were prepared as described above and handled in the same manner as for the immunohistochemical analysis, with the exception of prefixing for 20 min in 1% glutaraldehyde in PBS at 4°C prior to the precipitation process. After the precipitation, the slides were washed in PBS and postfixed for 30 min with 1% osmium tetroxide in PBS. The specimens were then dried in a graded ethanol series (50%, 70%, 80%, 90%, 100%) and acetone for 15 min at each concentration. After embedding in Epok 812 (Oken Shoji) and polymerization for 4 days at 60°C, ultrathin sections were cut on a microtome and stained with lead citrate for 2 min prior to EM.

RESULTS

At E17–18, the presence of stem cells in the donor mesencephalic neural tube was confirmed with nestin and Musashi-1 staining and transmission EM (Fig. 1). Positive staining was seen in all specimens for markers of neural precursors, including stem cells, nestin (Fig. 1A), and Musashi-1 (Fig. 1B). The nestin- and Musashi-1-positive stem cells at this stage had formed a neural tube and exhibited a pseudostratified neuroepithelial appearance. Double staining was carried out for Tuj-1 in addition to nestin (Fig. 1A), and the presence of some Tuj-1-positive cells indicated that some stem cells had already differentiated into cells of a neuronal lineage. The EM findings showed typical immature morphology with a very high nucleus to cytoplasm ratio (N/C ratio) and the absence of organelles in the cytoplasm (Fig. 1C).

No deaths from transplantation procedures or tumor formation of the grafted tissue were seen in any of the host

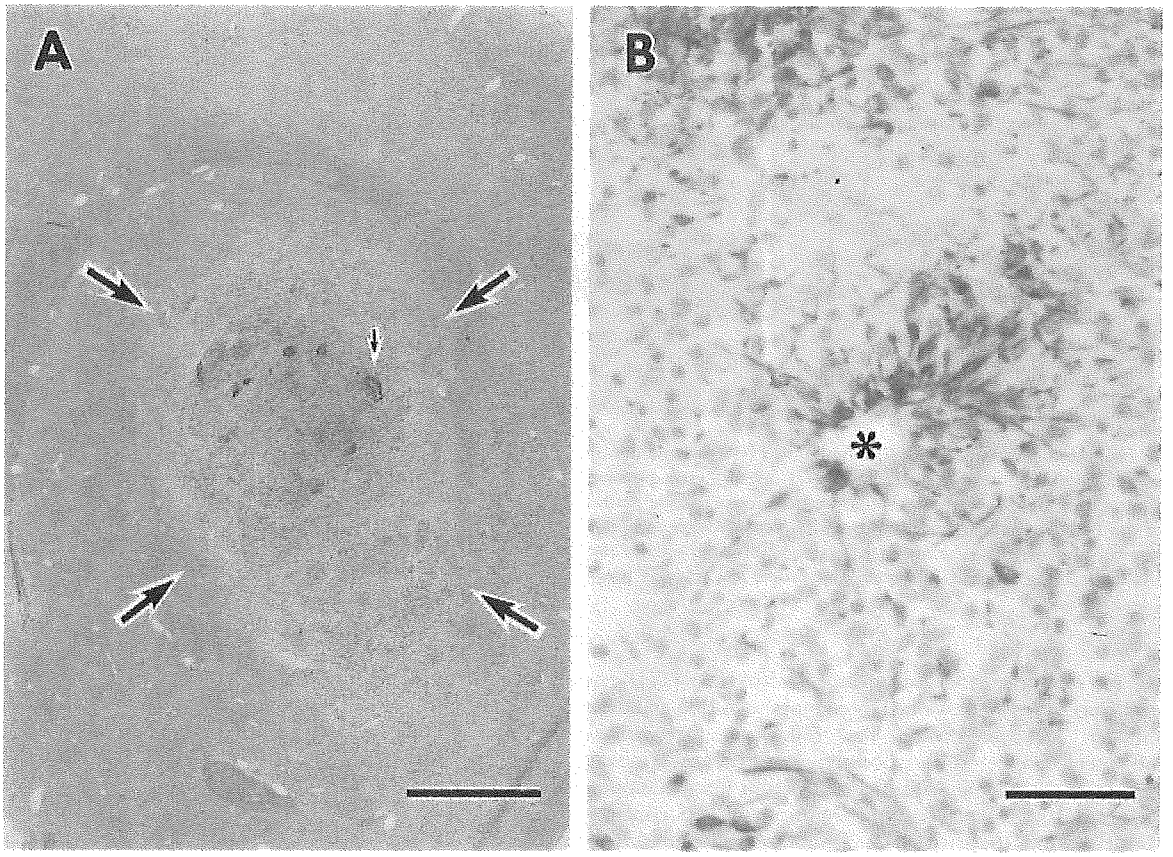

Fig. 2. H-E- and nestin-stained host specimens 45 days postimplant. **A:** H-E staining. The donor tissue can be clearly discriminated from the host by the appearance of rosette-forming (small arrow indicates one of the rosettes) and nonrosette-forming cells, but with diffusion into the host tissue indicating survival of the transplanted tissue in a xenogeneic environment (large arrows). **B:** Nestin staining. At a higher magnification, an example of a true rosette (asterisk) is seen, with all the rosette-forming cells staining positively for nestin. Some nestin-positive nonrosette-forming cells can be seen in the graft tissue surrounding the rosette. Scale bars = 1 mm in A; 50 μm in B.

rats, and all of the grafts taken at 4–8 weeks posttransplant survived well in the host xenogeneic environment. H-E staining in 45 day posttransplant specimens taken from the host animals revealed a discrete grafted mass in the striatum of the host brain (Fig. 2A), containing many rosettes in addition to other nonrosette-forming cells, indicating that the grafted tissue had survived in a xenogeneic graft environment, maintaining the ability to form both immature and differentiated cells. This was further demonstrated with the nestin staining, which revealed nestin-expressing cells in the rosettes but also showed many nestin-nonexpressing cells and some nestin-expressing cells in the nonrosette-forming cells surrounding the rosettes (Fig. 2B). The presence of nestin-expressing cells in and around true rosettes indicated that the self-renewal capability of the NESCs was maintained when they were transplanted in the xenogeneic adult brain environment. The presence of many nestin-negative cells surrounding the rosette suggests the capability of the nestin-positive immature cells within the rosette to produce mature cells, mimicking the normal development of the neural tube.

To evaluate the differentiation capability of the NESCs, cryostat sections of the grafts were further stained immunohistochemically using the pig-specific neurofilament and TH antibodies. Many pig-specific neurofilament-positive cells with a few short processes could be seen in nonrosette-forming cells at 45 days after the transplantation (Fig. 3A). Some TH-positive cells could be seen in the 45-day-old graft in nonrosette-forming cells, which also had a few short processes (Fig. 3B). The presence of pig-specific neurofilament- and TH-expressing cells surrounding the rosettes indicated the differentiation capability of the grafted pig NESCs, although these cells were still morphologically immature, with a few short processes.

An EM study was performed to assess synapse formation of donor-derived neurons (Fig. 4). A portion of an immature donor-derived cell could be seen containing polysomes, exhibiting a short process with clear spherical

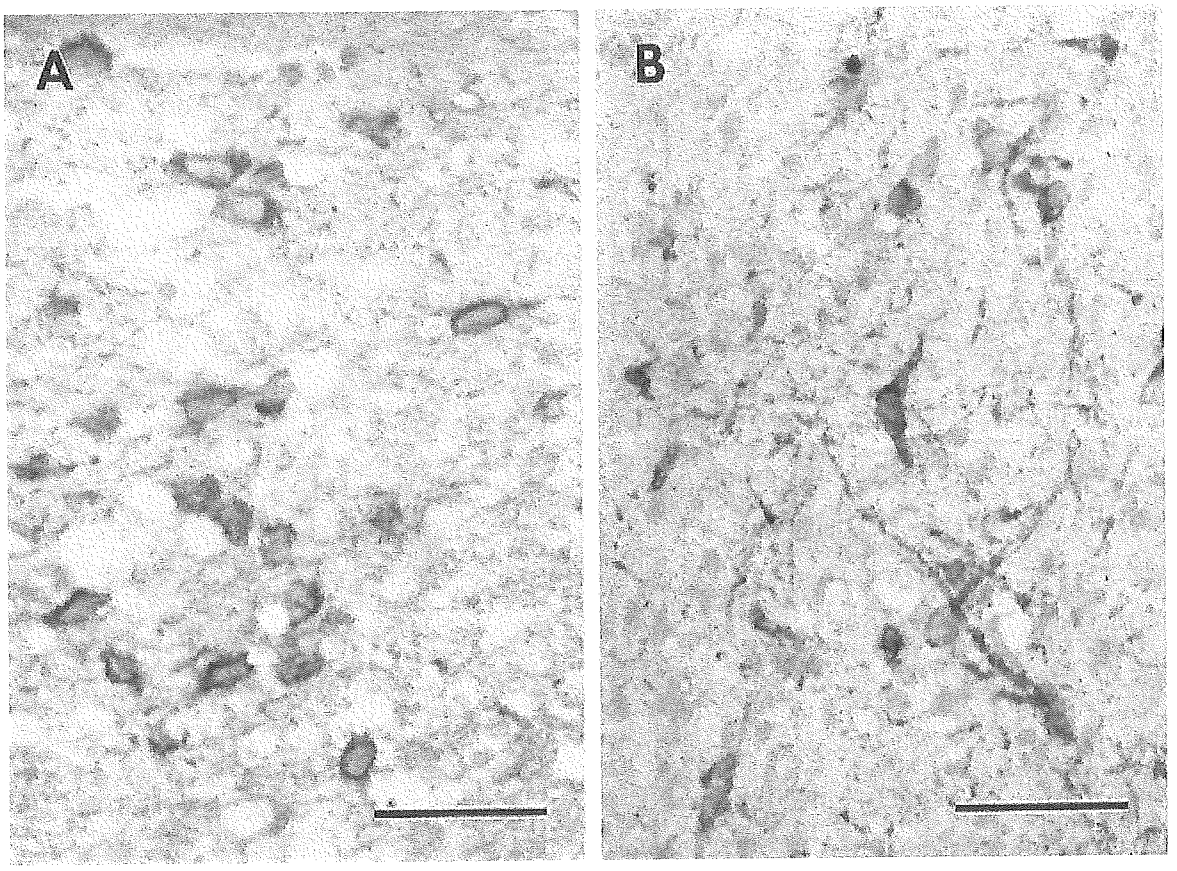

Fig. 3. Immunohistochemical findings of the grafted area 45 days postimplant. **A:** Pig-specific neurofilament staining shows positive neuronally differentiated cells but with a high N/C ratio and comparative lack of processes, indicating immature cell morphology. **B:** TH staining reveals positive donor-derived catecholaminergic cells in the graft. Although the cells demonstrate an immature morphology, processes are clearly visible. Scale bars = 50 μm.

vesicles demonstrating efferent synaptic formation with another host-derived mature cell without polysomes at 31 days after the transplantation (Fig. 4A). Afferent synapse formation in a cell staining positively for TH is demonstrated in Figure 4B. Two TH-negative axon terminals containing clear spherical vesicles contacted a TH-positive donor-derived cell at 45 days postimplant. An afferent synapse in the donor cell staining positively for the pig-specific neurofilament antibody was observed, whereas the terminal with clear spherical vesicles contacting the donor-derived neuron stained negatively against pig-specific neurofilament antibody at 45 days after the transplantation (Fig. 4C).

In immuno-EM using the pig-specific neurofilament antibody, many myelinated axons could be seen with both positive and negative staining for pig-specific neurofilament antibody in the grafted area at the EM level (Fig. 5), indicating that myelynation could be formed on the donor-derived axons in the xenogeneic environment. It remains to be elucidated, however, whether the myelin is formed by host- or donor-derived oligodendrocytes.

DISCUSSION

Neural stem cells are expected to be a clinically applicable donor material for neural grafting (Uchida and Toya, 1996; Studer et al., 1998; Sawamoto et al., 2001). Accumulating evidence has been clarifying the existence of various kinds of neural stem cells within the fetal and adult central nervous system (Lillien, 1998; Doetsch et al., 1999; Johansson et al., 1999; Momma et al., 2000; Keyoung et al., 2001). These cells have both self-renewal and multipotency as common characteristics and are thought to be of central nervous system origin. Stem cells divide asymmetrically into, for example, differentiated neural cells and daughter stem cells. This is the characteristic of self-renewal and multipotency. Logically, this should mean that fetal neural stem cells are identical to neural stem cells in the adult brain. This, however, is not so; adult

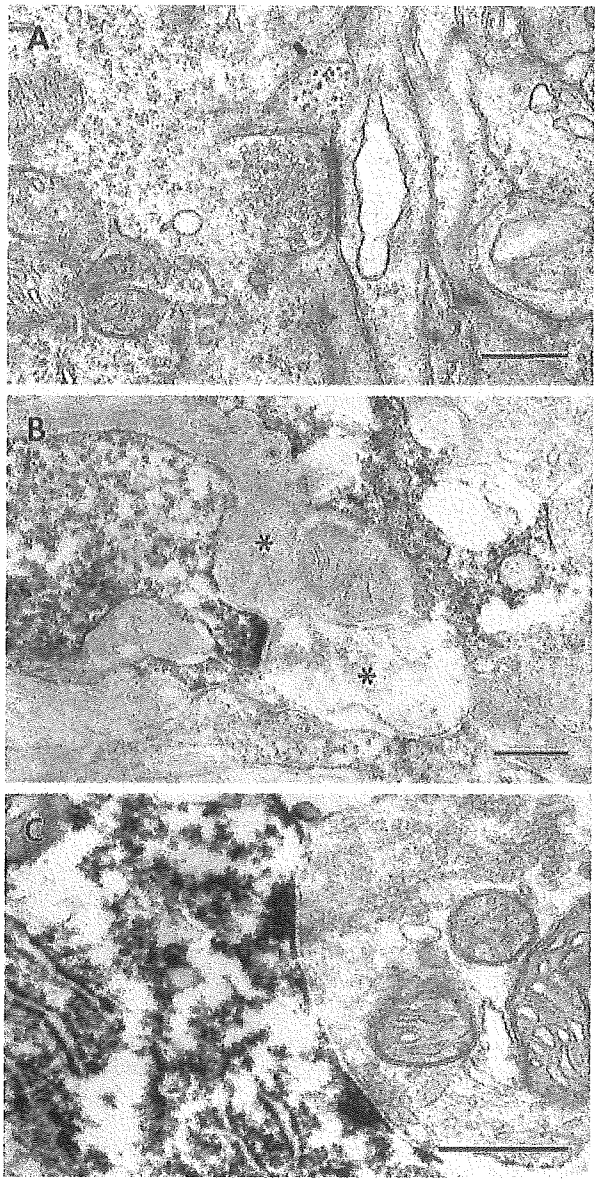

Fig. 4. EM and immuno-EM of the grafted area. **A:** EM at 31 days postimplant reveals an efferent synapse with many clear spherical vesicles and electrodense material from the polysome-containing immature cell on the left to the nonpolysome-containing cell on the right. By implication, the cell on the left is donor derived, whereas that on the right is host derived. **B:** Immuno-EM using TH antibody at 45 days postimplant shows axon terminals (asterisks) with vesicles contacting a darkly stained TH-positive dendrite. **C:** Immuno-EM using pig-specific neurofilament antibody at 45 days postimplant clearly illustrates an afferent synapse from the negative terminal with vesicles on the right to the darkly stained positive, and therefore donor-derived, cell on the left. Scale bars = 0.5 μm.

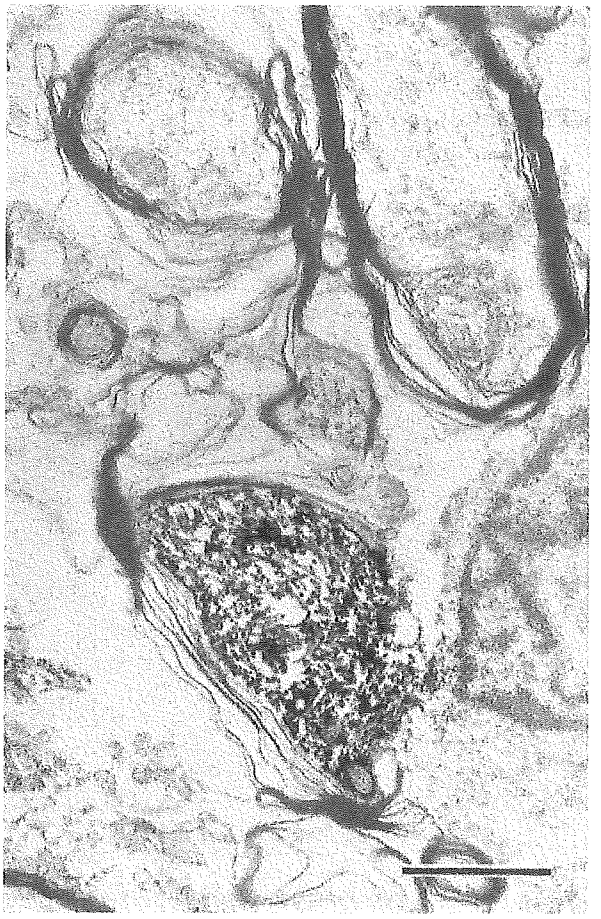

Fig. 5. Immuno-EM using pig-specific neurofilament antibody at 45 days postimplant demonstrates the presence of both positively and negatively stained myelinated axons in the graft. Scale bar = 1 μm.

neural stem cells have a higher probability of differentiating into astrocytes rather than neurons (Gritti et al., 1996; Palmer et al., 1999). In the case of a diseased brain, the more neurons that can be produced from the grafted stem cells the better, because of the importance of the reconstruction of the neural circuitry to arrive at functional repair. There are a variety of aims in transplantation therapy. However, our transplantation therapy is directly aimed at the regeneration of damaged neural circuitry through the unique property of NESCs of differentiating into neurons rather than other neural phenotypes, and this can be achieved only through the transplantation technique.

Our group has been concentrating on NESCs, which have a much higher probability of differentiating into neurons rather than astrocytes (Uchida et al., 1995, 1999; Hara et al., 2000). Qian et al. (2000) demonstrated that NESCs exhibited a distinct order of neuroblast production initially using the stem cell culture from the

embryonic mouse cerebral cortex. Human fetal NESCs, thus, would be the ideal donor material for supplying a large number of neurons into the damaged host brain and reconstructing the neuronal circuitry. Ethical considerations, however, restrict the use of human fetal tissue. In actual fact, practical considerations also rule out the use of human fetal NESCs, because the amount that can be harvested from each fetus is impractically small. Therefore, we turned our attention to readily available sources of practical amounts of fetal NESCs. Pig tissues are routinely used in modern medicine (Konigova et al., 2000; Khan et al., 2001), and in fact the use of transplanted pig neural tissues has already been reported for human beings in treating Parkinson's disease (Deacon et al., 1997; Schumacher et al., 2000). Although later-stage (after E25) porcine fetus neural tissue was used, the feasibility of porcine neural xenograft was recently evaluated in an animal model (Isacson et al., 1995; Isacson and Deacon, 1996; Armstrong et al., 2001; Larsson et al., 2001). Success with preliminary experiments using pig tissues led to the development of the transgenic pig to minimize the rejection response (Fodor et al., 1994; Cozzi and White, 1995). Pig fetuses, therefore, do not present the ethical problems associated with human fetuses as far as tissue transplantation is concerned. Furthermore, sows typically have large litters, so the practicality of obtaining a useful amount of grafting material is good. Given this abundant supply of fetal tissue with no ethical problems, and given that pig brain develops in a manner not dissimilar to the human brain, we wondered whether fetal pig NESCs would be a suitable transplant material for use in the damaged human brain. In this study, we examined grafted pig NESCs, focusing on whether xenogeneic neurons can form both efferent and afferent synapses with each other. It has been suggested that the host brain that has been somehow damaged prior to the grafting procedure presents a more favorable environment for the grafted tissue because of the possible presence of trophic factors induced by the damage (Nieto-Sampedro et al., 1984; Bakay et al., 1988). Parkinsonian model rats were therefore used as recipients for the xenograft because of the greater possibility of success of the transplant in the damaged host brain environment as a result of the reported better survival and process extension of donor-derived dopamine neurons in the Parkinsonian rat brain environment (Doucet et al., 1990; Nishino et al., 2000; Yurek and Fletcher-Turner, 2002). Evaluation of functional recovery was not the aim of the present study but will be addressed in the future, based on the success of the current study.

At the initial stage of our study, we tried to determine an appropriate gestation date in order to get viable pig NESCs. One unique aspect of our study was the use of nestin and Musashi-1 antibodies, originally developed by us, to confirm the presence of stem cells in the donor material. The gestation date at which early embryos were consistently obtained by cesarean section was from E17. EM findings show a typical pseudostratified neuroepithelial appearance and immature morphology of the cells at this stage, and nestin staining offered us good confirmatory evidence of the abundant presence of stem cells in addition to the Musashi-1 marker (Hockfield and McKay, 1985; Sakakibara et al., 1996; Kaneko et al., 2000). Thus, the mesencephalic neural tube region of E17 or E18 pig fetus was used for the stem cell source. In fact, the number of NESCs harvested from the neural tube of a single pig fetus was still very small, but the large litters commonly birthed by sows gave us a large number of neural tubes and a sufficient amount of NESCs to allow grafting in the small Parkinsonian model rat brain. Larger brains, such as in human beings, will obviously require much greater numbers of NESCs, so in vitro amplification techniques will have to be developed to provide appropriate numbers. Studies on such techniques are underway in our institute (Hayashi et al., 2002).

In the present study, both rosette- and nonrosette-forming cells coexisted in the implanted site. Rosette-forming cells denote immature cells with a CNS-related lineage that can be expected to form neural tissues as the cells mature (Kawata et al., 1991; Ohira et al., 1994). Thus, the asymmetrical division of the NESCs, including self-renewal, was paralleled in the xenogeneic graft environment just as if the NESCs were in situ. The fact that the rosette-forming cells were all positive for nestin confirms that the rosette formation was the result of self-renewal of the grafted NESCs. In the area surrounding the rosettes, nestin-positive nonrosette-forming cells were also visible, although the mechanism responsible for the rosette formation remains to be elucidated. As for the nonrosette-forming cells, staining with pig-specific antineurofilament antibody revealed the presence of donor-derived neurons in the graft. Some TH-positive cells were also seen in the graft, suggesting that, in addition to the ability to form neurofilaments, the cells could also express a neurochemical marker. Interestingly, the shape of the antibody-positive neurons was immature, with few processes. We supposed that pig NESCs would take longer to achieve the morphology of mature cells even though as immature cells they were still expressing neurofilaments, which is of considerable interest, because the gestation period for the pig is very long, 114 days. In other words, the cellular clocks of the pig will run at slower speeds and thus cells will obey their specific internal time clock even in a xenogeneic grafted environment. An important consideration is that both of the characteristics intrinsic to stem cells, i.e., self-renewal and multipotency, were clearly demonstrated in the graft, echoing the behavior of normally developing stem cells in situ.

An important observation for the xenografting related to the capacity of the transplanted NESCs to differentiate into neurons with synapses to or from the xenogeneic host brain in the transplant environment. One problem facing transplant researchers is confirming definitely which tissues are host-derived and which are donor-derived. Technically, it is almost impossible to mark all the donor cells, leading to ambiguity in the differentiation between host-derived and donor-derived tissue. It is thus

very difficult, when considering synaptic formation, to demonstrate clearly that a given synaptic formation is truly donor to host or host to donor rather than donor to donor. On the other hand, clear differentiation can be seen between immature donor cells and mature host cells because of the presence of polysomes in the former (Greenough et al., 2001). The presence of TH-positive cells in the striatum is also a clear indicator of donor-derived cells, but some donor-derived cells are not TH positive, thus leading to the same problem of host–donor ambiguity. Species-specific neurofilament identification, however, removes ambiguity and clearly demonstrates which cells are donor-derived or host-derived.

Polysome-containing immature cells were revealed in the graft by the EM, yet these cells were capable of forming synapses. The model we were using was an adult rat, in which such immature cells are not found. Thus the polysome-containing cells seen in the graft area were donor derived. Although we are not certain whether the target cells were donor derived or host derived, the lack of polysomes in these target cells might suggest that they are host derived. Further research is required to elucidate the origin of the target cells. A further, somewhat surprising finding was that polysome-containing cells, which are morphologically immature, were capable of synaptic formation, which is a characteristic of mature neurons. Likewise, we observed immunohistochemically that cells with few processes, a characteristic of immature neurons, were expressing neurofilaments in the same way as mature neurons would. TH-positive cells, which also have a few processes, were found in the graft. Because TH-positive neurons are not present in the host Parkinsonian rat striatum, they must be donor derived. This shows that pig NESCs are capable of differentiation into catecholamine-producing neurons. We further detected afferent synapses in the TH-positive cells by immuno-EM. These findings show that TH-positive donor-derived cells with immature morphology are capable of receiving synapses. Immuno-EM using pig-specific neurofilament antibody revealed clearly the synaptic contact from the host striatal cell to the donor-derived neuron. These EM results indicate that pig stem cell-derived neurons can make both efferent and afferent synaptic contacts with xenogeneic host derived neurons.

In the valid reconstruction of damaged neural circuits, myelinated fibers are of primary importance, together with synapse formation. Our immunohistochemical EM findings revealed the myelination around the axons staining positively for pig-specific neurofilament antibody, i.e., donor-derived myelinated axons, together with host-derived myelinated axons that did not stain positively for pig-specific antibody. Thus, pig NESCs are capable of, among other useful properties, differentiation into neurons with myelinated axons. The origin of the myelin, however, remains to be confirmed. The oligodendrocytes responsible for the myelin formation may be either donor derived or host derived or perhaps a combination of the two. Donor-derived oligodendrocytes would be the result of the multipotency of the neural stem cells, which is an exciting possibility, insofar as it adds to the attractiveness of neural stem cell transplantation. Not only would the grafted stem cells form axons but they would also be capable of providing all the supporting cells necessary for repairing damaged neural circuitry. However, it is also an exciting possibility that the host environment is capable of myelinating donor-derived axons. It will be important to ascertain which of these mechanisms is the actual one. If a xenogeneic host environment is capable of myelinating donor-derived axons, then neuronal transplantation techniques using differentiated neurons will be sufficient. On the other hand, if the xenogeneic host environment is incapable of such myelinization, then stem cell transplantation, with multipotency allowing differentiation into all cell types required, will offer many advantages.

With all of these factors taken into consideration, pig NESCs are capable of surviving in a xenogeneic environment, differentiating into neurons that can express neurochemical markers such as TH, which form valid synapses with xenogeneic host neurons and myelinated axons. In short, pig NESCs, after further and much longer term studies, may well be a viable alternative donor material for the physiological repair of compromised neural circuits. As a next step after the present study, much longer term assessments will be required to address the issues of functional recovery of the grafted animals and aspects concerning safety (e.g., tumor formation in the graft and viral infection of swine origin to the host). These investigations are essential when considering the clinical application of swine NESCs in the repair of damaged neuronal circuitry, including their application in human beings. However, as a first step, the present study successfully demonstrates the key points of survival of swine NESCs in the xenogeneic grafted environment, with myelinated axon formation and both types of synaptic formation with the xenogeneic host neurons.

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REFERENCES

Armstrong RJE, Harrower TP, Hurelbrink CB, Mclaughin M, Ratcliffe EL, Tyers P, Richards A, Dunnett SB, Rosser AE, Barker RA. 2001. Porcine neural xenografts in the immunocompetent rat: immune response following grafting of expanded neural precursor cells. Neuroscience 106:201–216.

Bakay RAE, Fiandaca MS, Sweeney KM, Colbassani HJ, Collins DC. 1988. Delayed stereotactic transplantation technique in non-human primates. In: Gash DM, Sladek JR, editors. Progress in Brain Research. New York: Elsevier Science Publishers BV. p 463–471.

Cozzi E, White DJG. 1995. The generation of transgenic pigs as potential organ donors for humans. Nat Med 1:964–966.

Deacon T, Schumacher J, Dinsmore J, Thomas C, Palmer P, Kott S, Edge A, Penney D, Kassissieh S, Dempsey P, Isacson O. 1997. Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. Nat Med 3:350–353.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703–716.

Doucet G, Brundin P, Descarries L, Björklund A. 1990. Effect of prior dopamine denervation on survival and fiber outgrowth from intrastriatal fetal mesencephalic grafts. Eur J Neurosci 2:279–290.

Fisher LJ. 1997. Neural precursor cells: applications for the study and repair of the central nervous system [review]. Neurobiol Dis 4:1–22.

Fodor WL, Williams BL, Matis LA, Madri JA, Rollins SA, Knight JW, Velander W, Squinto SP. 1994. Expression of a functional human complement inhibitor in a transgenic pig as a model for the prevention of xenogeneic hyperacute organ rejection. Proc Natl Acad Sci USA 91: 11153–11157.

Greenough WT, Klintsova AY, Irwin SA, Galvez R, Bates KE, Weiler IJ. 2001. Synaptic regulation of protein synthesis and the fragile X protein. Proc Natl Acad Sci USA 98:7101–7106.

Gritti A, Parati EA, Cova L, Frolichsthal P, Galli R, Wanke E, Faravelli L, Morassutti DJ, Roisen F, Nickel DD, Vescovi AL. 1996. Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. J Neurosci 16:1091–1100.

Hara K, Uchida K, Fukunaga A, Kuroshima Y, Yamada M, Kawase T. 2000. Neurite growth capability of rat fetal neuronal cells against matured CNS myelin in vitro. Cell Transplant 9:717–724.

Hayashi T, Uchida K, Mine Y, Yamada M, Kawase T. 2002. Feasibility of using early mesencephalic neural plate for intracerebral grafting. Cell Transplant 11:465–470.

Hockfield S, McKay RD. 1985. Identification of major cell classes in the developing mammalian nervous system. J Neurosci 5:3310–3328.

Isacson O, Deacon TW. 1996. Specific axon guidance factors persist in the adult brain as demonstrated by pig neuroblasts transplanted to the rat. Neuroscience 75:827–837.

Isacson O, Deacon TW, Pakzaban P, Galpern WR, Dinsmore J, Burns LH. 1995. Transplanted xenogeneic neural cells in neurodegenerative disease models exhibit remarkable axonal target specificity and distinct growth patterns of glial and axonal fibers. Nat Med 1:1189–1194.

Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisen J. 1999. Identification of a neural stem cell in the adult mammalian central nervous system. Cell 96:25–34.

Kaneko Y, Sakakibara S, Imai T, Suzuki A, Nakamura Y, Sawamoto K, Ogawa Y, Toyama Y, Miyata T, Okano H. 2000. Musashi 1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. Dev Neurosci 22:139–153.

Kawata M, Sekiya S, Kera K, Kimura H, Takamizawa H. 1991. Neural rosette formation within in vitro spheroids of a clonal human teratocarcinoma cell line, PA-1/NR: role of extracellular matrix components in the morphogenesis. Cancer Res 51:2655–2669.

Keyoung HM, Roy NS, Benraiss A, Louissaint A Jr, Suzuki A, Hashimoto M, Rashbaum WK, Okano H, Goldman SA. 2001. High-yield selection and extraction of two promoter-defined phenotypes of neural stem cells from the fetal human brain. Nat Biotech 19:843–850.

Khan SS. Trento A, DeRobertis M, Kass RM, Sandhu M, Czer LS, Blanche C, Raissi S, Fontana GP, Cheng W, Chaux A, Matloff JM. 2001. Twenty-year comparison of tissue and mechanical valve replacement. J Thorac Cardiovasc Surg 122:257–269.

Konigova R, Matouskova E, Broz L. 2000. Burn wound coverage and burn wound closure. Acta Chir Plast 42:64–68.

Larsson LC, Frielingsdorf H, Mirza B, Hansson SJ, Anderson P, Czech KA, Strandberg M, Widner H. 2001. Porcine neural xenografts in rats and mice: donor tissue development and characteristics of rejection. Exp Neurol 172:100–114.

Lillien L. 1998. Neural progenitors and stem cells: mechanisms of progenitor heterogeneity. Curr Opin Neurobiol 8:37–44.

Momma S, Johansson CB, Frisen J. 2000. Get to know your stem cells. Curr Opin Neurobiol 10:45–49.

Murase N, Starzl TE, Demetris AJ, Valdivia L, Tanabe M, Cramer D, Makowka L. 1993. Hamster-to-rat heart and liver xenotransplantation with FK506 plus antiproliferative drugs. Transplantation 55:701–708.

Nieto-Sampedro M, Whittemore SR, Needels DL, Larson J, Cotman CW. 1984. The survival of brain transplants is enhanced by extracts from injured brain. Proc Natl Acad Sci USA 81:6250–6254.

Nishino H, Hida H, Takei N, Kumazaki M, Nakajima K, Baba H. 2000. Mesencephalic neural stem (progenitor) cells develop to dopaminergic neurons more strongly in dopamine-depleted striatum than in intact striatum. Exp Neurol 164:209–214.

Ohira A, Yamamoto M, Honda O, Ohnishi Y, Inomata H, Honda Y. 1994. Glial-, neuronal- and photoreceptor-specific cell markers in rosettes of retinoblastoma and retinal dysplasia. Curr Eye Res 13:799–804.

Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH. 1999. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. J Neurosci 19:8487–8497.

Qian X, Shen Q, Goderie SK, He W, Capela A, Davis AA, Temple S. 2000. Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron 28:69–80.

Reynolds BA, Weiss S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710.

Sakakibara S, Imai T, Hamaguchi K, Okabe M, Aruga J, Nakajima K, Yasutomi D, Nagata T, Kurihara Y, Uesugi S, Miyata T, Ogawa M, Mikoshiba K, Okano H. 1996. Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell. Dev Biol 176:230–242.

Sawamoto K, Nakao N, Kakishita K, Ogawa Y, Toyama Y, Yamamoto A, Yamaguchi M, Mori K, Goldman SA, Itakura T, Okano H. 2001. Generation of dopaminergic neurons in the adult brain from mesencephalic precursor cells labeled with a nestin-GFP transgene. J Neurosci 21:3895–3903.

Schumacher JM, Ellias SA, Palmer EP, Kott HS, Dinsmore J, Dempsey PK, Fischman AJ, Thomas C, Feldman RG, Kassissieh S, Raineri R, Manhart C, Penney D, Fink JS, Isacson O. 2000. Transplantation of embryonic porcine mesencephalic tissue in patients with PD. Neurology 54:1042–1050.

Stemple DL, Mahanthappa NK. 1997. Neural stem cells are blasting off. [minireview]. Neuron 18:1–4.

Studer L, Tabar V, McKay RD. 1998. Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. Nat Neurosci 1:290–295.

Uchida K, Toya S. 1996. Grafting of genetically manipulated cells into adult brain: toward graft-gene therapy [review]. Keio J Med 45:81–89.

Uchida K, Tsuzaki N, Nagatsu T, Kohsaka S. 1992. Tetrahydrobiopterin-dependent functional recovery in 6-hydroxydopamine-treated rats by intracerebral grafting of fibroblasts transfected with tyrosine hydroxylase cDNA. Dev Neurosci 14:173–180.

Uchida K, Kawaja MD, Toya S, Roach AH. 1995. Transgenic neural plate contributes neuronal cells that survive greater than one year when transplanted into the adult mouse central nervous system. Exp Neurol 132: 194–208.

Uchida K, Roach AH, Kawaja MD, Toya S. 1999. Successful survival of grafted transgenic neural plate cells in adult central nervous system environment. Cell Mol Neurobiol 19:79–86.

Yurek DM, Fletcher-Turner A. 2002. Temporal changes in the neurotrophic environment of the denervated striatum as determined by the survival and outgrowth of grafted fetal dopamine neurons. Brain Res 931: 126–134.

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Hepatocyte growth factor promotes proliferation and neuronal differentiation of neural stem cells from mouse embryos

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Abstract

Hepatocyte growth factor (HGF), originally cloned as a hepatocyte mitogen, has recently been reported to exhibit neurotrophic activity in addition to being expressed in different parts of the nervous system. At present, the effects of HGF on neural stem cells (NSCs) are not known. In this study, we first report the promoting effect of HGF on the proliferation of neurospheres and neuronal differentiation of NSCs. Medium containing only HGF was capable of inducing neurosphere formation. Addition of HGF to medium containing fibroblast growth factor 2 or epidermal growth factor increased both the size and number of newly formed neurospheres. More neurons were also obtained when HGF was added in differentiation medium. In contrast, neurosphere numbers were reduced after repeated subculture by mechanical dissociation, suggesting that HGF-formed neurospheres comprised predominantly progenitor cells committed to neuronal or glial lines. Together, these results suggest that HGF promotes proliferation of neurospheres and neuronal differentiation of NSCs derived from mouse embyos.

Introduction

Neural stem cells (NSCs) possess the ability to self-renew and give rise to various types of neurons, astrocytes, and oligodendrocytes in vitro and may thus play a major role in both mammalian central nervous system (CNS) development and function throughout adulthood (Reynolds and Weiss, 1992, 1996; Temple and Davis, 1994; Palmer et al., 1997). Control of the proliferation and differentiation of NSCs may be important for the potential development of transplantation strategies and other therapeutic approaches for the treatment of neuronal injuries and neurodegenerative diseases (Svendsen et al., 1997; Armstrong et al., 2000; Okano, 2002). In vitro studies of NSC-based neurogenesis and gliogenesis suggest that these processes occur by stepwise restriction and are dependent on environmental signals (Ahmed et al., 1995; Tropepe et al., 1997, 1999; Arsenijevic and Weiss, 1998; Qian et al., 2000). A number of growth factors support the proliferation of NSCs and differentiation from their progenitors. Epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) play important roles in proliferation and maintenance of NSCs. Recently, other factors, such as ciliary neurotrophic factor (CNTF) and insulin-like growth factor 1 (IGF-1), have been reported to function as key players in control of proliferation and maintenance of NSCs (Arsenijevic et al., 2001; Shimazaki et al., 2001). FGF-2 and platelet-derived growth factor (PDGF) are also known to enhance neuronal differentiation (Johe et al., 1996; Erlandsson et al., 2001; Yoshimura et al., 2001). On the other hand, CNTF and bone morphogenetic protein

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(BMP) have been shown to enhance astrocyte differentiation in culture (Johe et al., 1996; Bonni et al., 1997; Shimazaki et al., 2001), whereas triiodothyronine (T3) has been shown to promote oligodendrocyte differentiation (Johe et al., 1996). FGF-2, CNTF, leukemia inhibitory factor (LIF), brain-derived neurotrophic factor (BDNF), and PDGF have been classified as neurotrophic factors and play essential roles in the development, maintenance, activity-dependent modulation, and regeneration of the nervous system. Hepatocyte growth factor (HGF) is a polypeptide growth factor that acts by binding to the c-Met tyrosine kinase receptor. HGF and c-Met have been found to be present in the developing and mature CNS (Jung et al., 1994; Honda et al., 1995; Hamanoue et al., 1996; Achim et al., 1997). HGF is a pleiotrophic cytokine that induces mitogenesis, motility, morphogenesis, and antiapoptotic activities, and has recently been shown to have neurotrophic ability. However, it is unknown whether HGF has any effects on the proliferation or differentiation of NSCs. In this study, we examined the effect of HGF on the proliferation and differentiation of NSCs isolated from E14 mouse striatal cells in vitro.

Results

Effects of HGF on neurosphere formation and proliferation of neural progenitors

Neurospheres were not observed 7 days postculture of primary E14 striatal cells at low density in the absence of growth factors (Fig. 1). A significant number of neurospheres (63.8 ± 44.8 cells/well) were observed at as low as 5 ng/ml HGF. The number of neurospheres increased in a dose-dependent manner until 20 ng/ml HGF and reached a plateau at 50 ng/ml (Fig. 1). The number of neurospheres formed by HGF was less than that by FGF-2, EGF, or their combination at any concentration (Fig. 1b). The addition of 20 ng/ml of HGF to FGF-2, EGF, or their combination, significantly increased the number of neurospheres (*without HGF*: FGF-2, 341.3 ± 89.6 cells/well; EGF, 146.3 ± 28.7 cells/well; FGF-2 + EGF, 507 ± 95.7 cells/well; *with HGF*: FGF-2, 745.9 ± 115.1 cells/well; EGF, 511.9 ± 43.5 cells/well; FGF-2 + EGF, 1218.8 ± 143.6 cells/well) (Fig. 1).

To determine the effects of HGF on the proliferation of neural progenitors, neurosphere size was measured using phase-contrast microscopy. Neurospheres were not observed in the absence of HGF. In the presence of 20 ng/ml HGF, neurospheres were observed, but they were smaller than those generated with the other growth factors. When HGF was added to the medium containing FGF-2, EGF, or their combination, neurosphere size increased significantly (Table 1).

To determine the effects of HGF on the formation of secondary and tertiary neurospheres, HGF-generated primary or secondary neurospheres were dissociated mechanically and again incubated with HGF at a density of 500 cells/200 μl in a 96-well dish. Seven days later, the number of tertiary generated neurospheres was much less smaller as

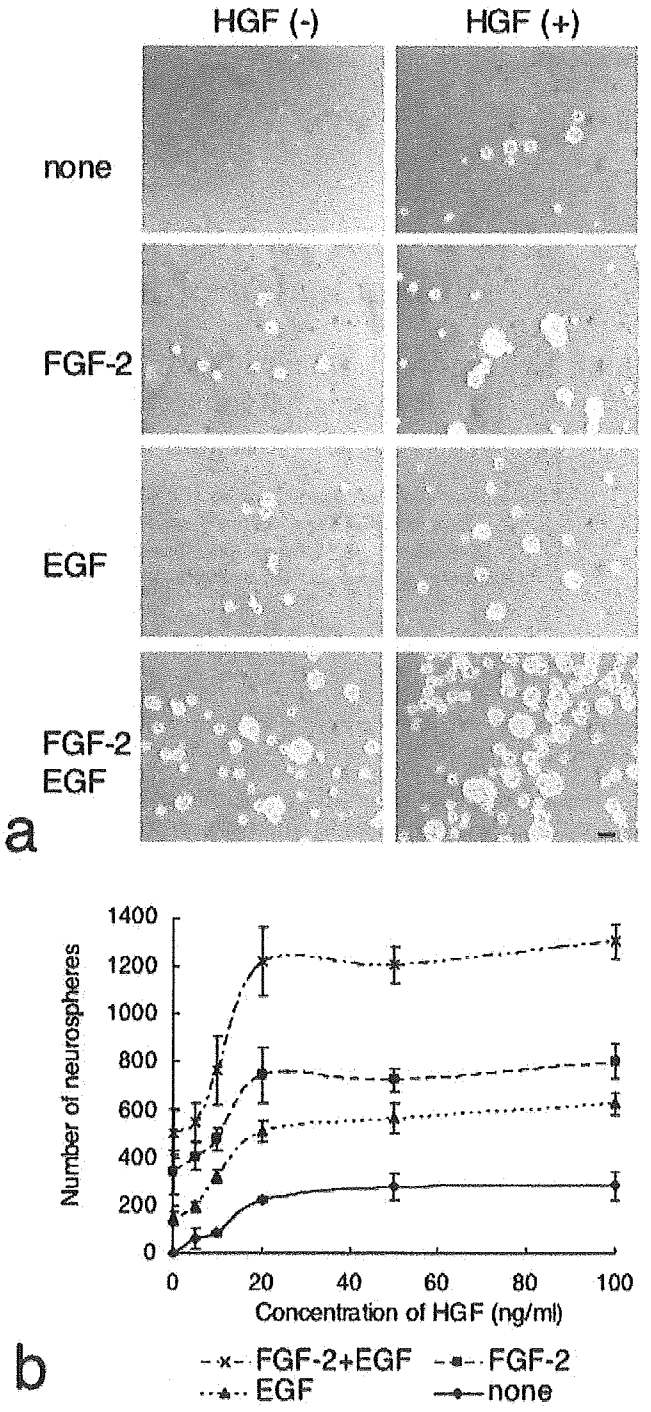

Fig. 1. Effects of HGF on the formation of neurospheres isolated from mouse E14 striatal cells. (a) Photographs of primary neurospheres in the presence of various growth factors for 7 days. Bar = 50 μm. (b) Graph showing number of neurospheres from E14 striatal cells. In the presence of FGF-2 (■), EGF (▲), FGF-2 plus EGF (×), or nothing (◆) and various concentrations of HGF, 75,000 cells per well were incubated in a 24-well plate for 7 days. Values represent means ± SEM of five independent experiments.

Table 1
Effects of HGF on the size and numbers of primary neurospheres[a]

	None	FGF-2		EGF		FGF-2 + EGF	
	+ HGF	− HGF	+ HGF	− HGF	+ HGF	− HGF	+ HGF
200 μm	50.1 ± 8.1	128.0 ± 12.1	413.3 ± 42.3*	90.9 ± 8.2	435.4 ± 82.2*	221.7 ± 44.2	809.2 ± 55.3*
100–200 μm	180.4 ± 50.1	768.3 ± 90.1	1395.0 ± 111.2*	590.9 ± 54.2	1306.2 ± 151.2*	886.8 ± 77.1	1402.5 ± 101.3*
≤100 μm	670.2 ± 88.2	653.7 ± 52.4	1291.7 ± 85.4*	818.2 ± 99.5	1088.5 ± 123.2*	1241.5 ± 99.2	1618.3 ± 96.3*
Total No.	900.7 ± 20.1	1750.0 ± 55.1	3100.0 ± 66.2*	1500.0 ± 65.1	2830.0 ± 86.4*	2350.0 ± 54.2	3830.0 ± 75.3*

[a] E14 striatal cells were plated at 3×10^5 cells per well in a six-well plate in the presence of the indicated growth factors. The size and number of primary neurospheres were determined 7 days later from counts obtained in five different experiments.
* $P < 0.05$ versus − HGF.

compared with the secondary neurospheres (Fig. 2a). Then, we next investigated the ability of the primary neurospheres to form secondary neurospheres in the presence of HGF, FGF-2 + EGF, or FGF-2 + EGF + HGF. The neurospheres generated by HGF, FGF-2 + EGF, or FGF-2 + EGF + HGF were dissociated mechanically, and the cells were further cultured in growth medium containing FGF-2 + EGF. Seven days later, the number of secondary neurospheres generated with the medium containing HGF or FGF-2 + EGF + HGF was smaller than that with FGF-2 + EGF (Fig. 2b). These results suggested that HGF increased the number and size of the neurospheres and that most of the cells in these neurospheres generated with HGF were neural progenitors. This hypothesis can explain the decrease in self-renewal ability of the cells proliferated by HGF treatment, leading to the formation of a decreased number of secondary neurospheres.

Character of the cells in the neurosphere obtained by HGF

Immunostaining to determine the expression of the HGF receptor c-Met was performed on both cells in the neurosphere and those dissociated in isolation with HGF. Most of the cells in the neurospheres and the dissociated cells from neurospheres expressed c-Met immunohistochemically (Figs. 3a, b). Expression of c-Met protein on neurospheres isolated with HGF or FGF-2 and EGF was also confirmed by Western blot analysis (data not shown). The immunopositivity for c-Met was also observed on the cells isolated with FGF-2, EGF, HGF, or their combination (data not shown). Cells from neurospheres isolated with 20 ng/ml HGF were also immunopositive for nestin, a stem cell marker (Fig. 3c).

Effect of HGF on BrdU incorporation of neural progenitors

To determine the mechanism underlying the proliferative effects of HGF on neural progenitors, neurospheres were co-incubated with 10 μM BrdU for 2 h and fixed. Addition of HGF to the medium containing FGF-2, EGF, or their combination significantly increased the percentage of BrdU-

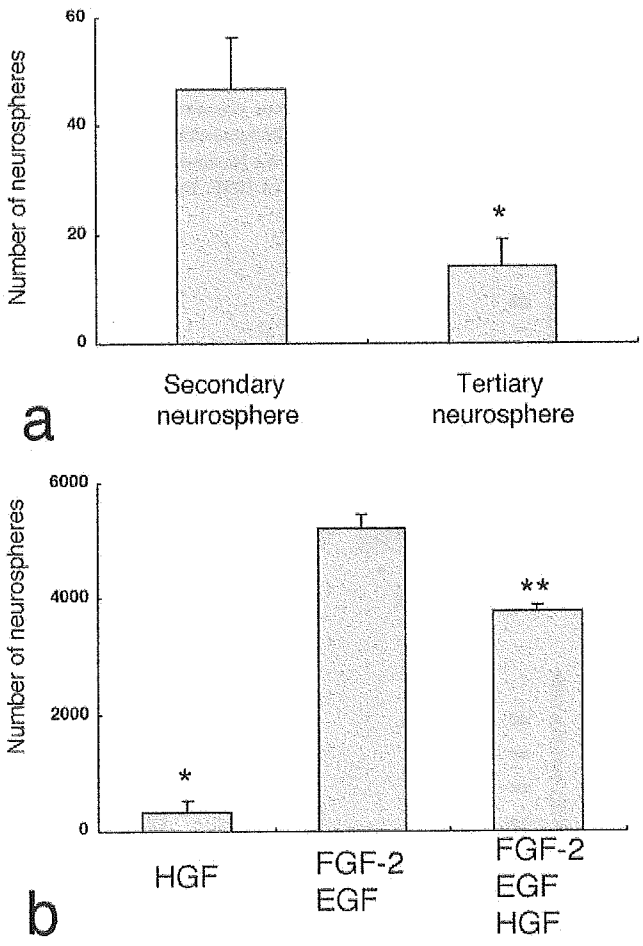

Fig. 2. Formation of secondary neurospheres generated by HGF or other growth factors. (a) HGF-generated primary or secondary neurospheres were collected, dissociated, and again incubated with HGF at a density of 500 cells/200 μl in a 96-well dish. The number of secondary and tertiary neurospheres was counted 7 days later (derived from the originally plated $500 \times 96 = 4.8 \times 10^4$ cells). All data are presented as means ± SEM of three independent experiments. *$P < 0.01$ versus secondary neurospheres. (b) Primary neurospheres (HGF-, FGF+EGF-, FGF+EGF+HGF-generated neurospheres) were collected, dissociated, and replated at 500 cells/200 μl in a 96-well dish. The number of secondary neurospheres was counted 7 days later (derived from the originally plated $500 \times 96 = 4.8 \times 10^4$ cells). All data are presented as means ± SEM of four independent experiments. * $P < 0.001$ and ** $P < 0.01$ versus FGF + EGF-generated neurospheres.

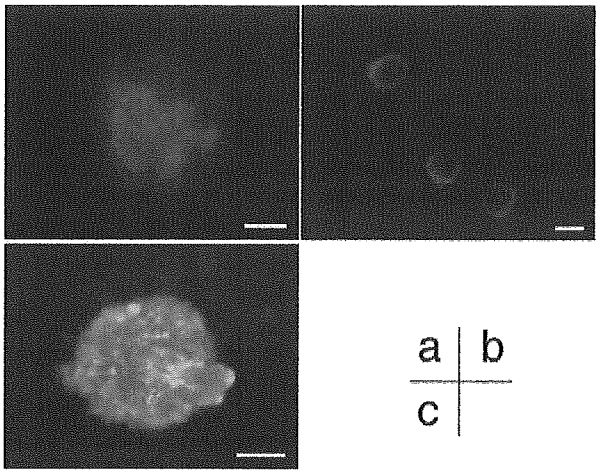

Fig. 3. Immunostaining of c-Met receptor (a, b) and nestin (c). (a) c-Met receptor expression (green) on the cells in the neurosphere cultured in the presence of FGF-2 and EGF. Cells were counterstained with Hoechst (blue) to show nuclei. Bar = 20 μm. (b) Immunostaining of c-Met receptor in single dissociated cells. Bar = 10 μm. (c) Nestin expression (red) on the cells in the neurosphere in the presence of HGF. Bar = 50 μm

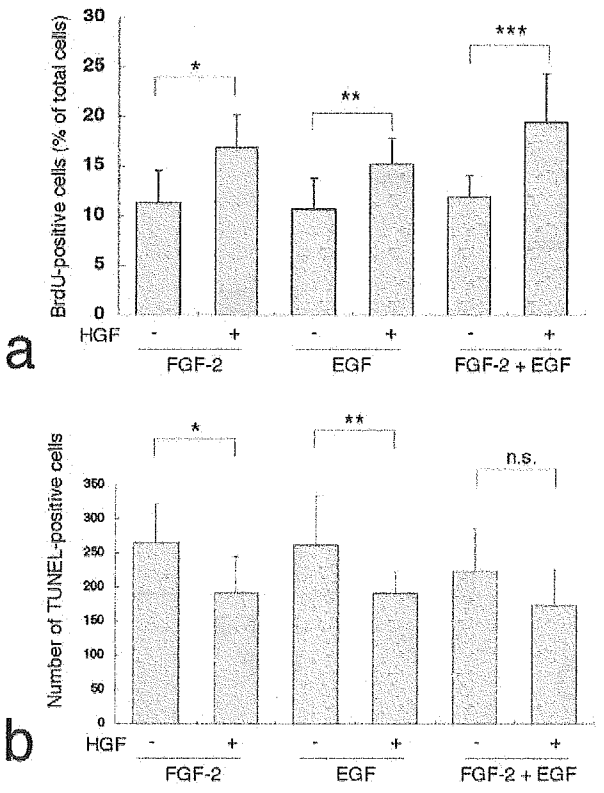

Fig. 4. (a) Percentage of BrdU-positive cells in the neurospheres cultured in the presence of various growth factors. BrdU (10 μM) was added to the secondary neurospheres for 2 h. Neurospheres were mechanically dissociated, plated in 24-well plates, and fixed 12 h later. Values represent means ± SEM of five independent experiments. $*P < 0.05$ versus FGF, $**P < 0.01$ versus EGF, $***P < 0.01$ versus FGF+EGF. (b) Numbers of TUNEL-positive cells in the neurospheres with/without HGF. Secondary neurospheres were mechanically dissociated, fixed in 1% paraformaldehyde, and stained using the TUNEL kit. Values represent means ± SEM of five independent experiments. $*P < 0.05$ versus FGF, $**P < 0.05$ versus EGF.

positive cells (Fig. 4a). There is another possibility that HGF promotes survival of neural progenitors by inhibiting cell death during culture. To explore the effects of HGF on the survival of neural progenitors, TUNEL staining was performed on neurospheres cultured in the medium with and without HGF. Addition of HGF decreased the number of TUNEL-positive cells in the neurosphere (Fig. 4b).

Effect of HGF on differentiation of neural progenitors

To elucidate the effect of HGF on the differentiation of neural progenitors, and also to verify whether neurospheres formed with HGF contain neural progenitors, their ability to differentiate into neurons, astrocytes, oligodendrocytes, or other cell types was investigated. First, the secondary neurospheres, which were cultured in the medium containing 20 ng/ml HGF for 7 days, were dissociated and plated on coverslips with 1% FBS with and without 20 ng/ml HGF for 7 days. The cells were immunostained with neuronal marker, MAP2 (red), glial marker, GFAP (green), and nuclear stain, Hoechst (blue) (Fig. 5). The immunopositive cells for each marker were counted and their percentages calculated. Interestingly, when HGF was added to the medium during differentiation, more neurons were obtained than astrocytes (*without HGF*: neurons, 39.8 ± 11.7 %; astrocytes, 38.0 ± 16.0%; *with HGF*: neurons, 52.5 ± 7.9%, astrocytes, 35.2 ± 8.9%) (Fig. 6a). Similar percentages were obtained when the cells were isolated with FGF-2 and EGF

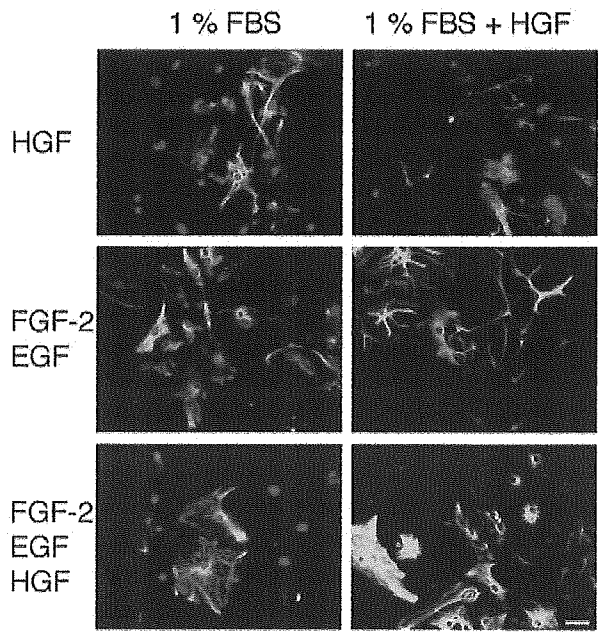

Fig. 5. Effect of HGF on the percentage of phenotypes. Double-labeled immunocytochemistry of cells from neurospheres in the presence of HGF, FGF-2 + EGF, and FGF-2 + EGF + HGF. The cells were plated in 1% FBS. or 1% FBS + HGF, MAP-2-positive neurons (red), GFAP-positive astrocytes (green), and Hoechst-labeled nuclei (blue) are shown. Bar = 50 μm.

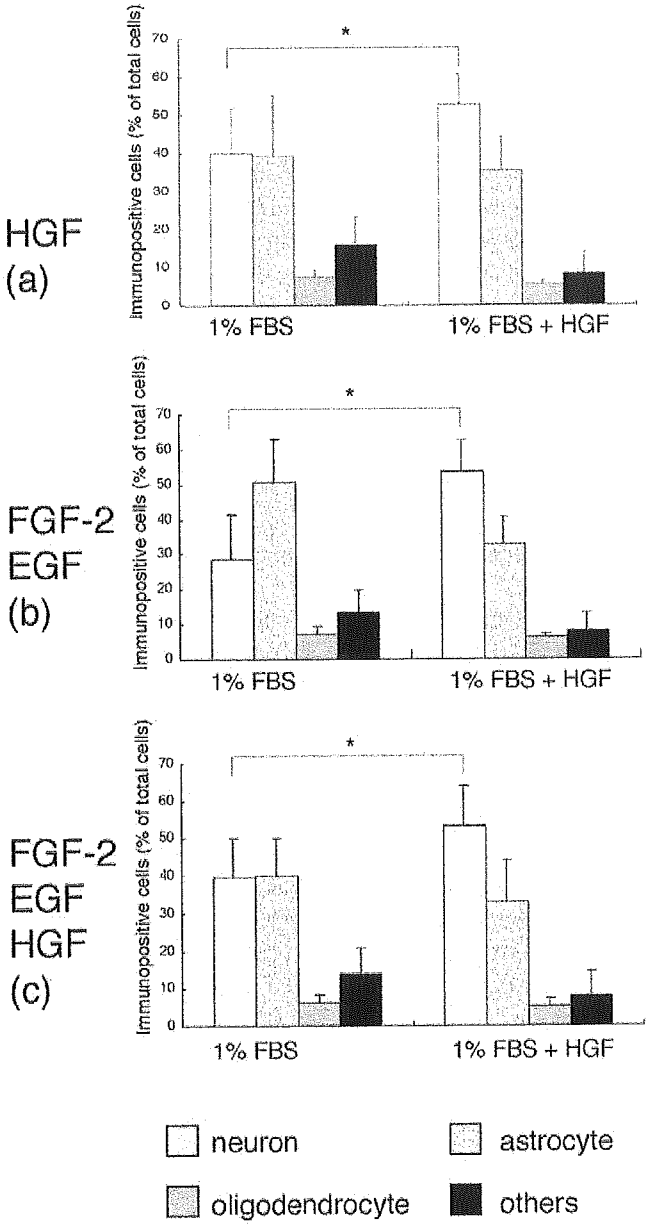

Fig. 6. Neurospheres cultured in the presence of HGF (a), FGF-2 + EGF (b), and FGF-2 + EGF + HGF (c) were differentiated in 1% FBS or 1% FBS plus HGF (20 ng/ml) for 7 days. Values represent means ± SEM of five independent experiments. *$P < 0.01$ versus 1% FBS neuron.

(*without HGF*: neurons, 28.5 ± 12.7%; astrocytes, 50.8 ± 11.9%; *with HGF*: neurons, 53.5 ± 8.9%; astrocytes, 32.6 ± 7.9%) (Fig. 6b). When 20 ng/ml HGF was added to the medium when primarily brain cells were cultured, there was a tendency to have more neurons after differentiation in the medium containing 1% FBS without HGF (Fig. 6c, left). However, when HGF was added to the medium containing 1% FBS for differentiation, a similar percentage of neurons was obtained (Fig. 6c, right). These results suggest that HGF promotes differentiation into neurons when it is added to the medium for differentiation.

Discussion

The establishment of culture systems for the expansion of NSCs using FGF-2 and/or EGF as the mitogen in vitro provides a useful model for examining cellular mechanisms underlying the development of the CNS (Reynolds and Weiss, 1992; Temple and Devis, 1994; Reynolds and Weiss, 1996; Weiss et al., 1996; McKay, 1997; Palmer et al., 1997). Knowledge of the mechanisms of NSC proliferation and differentiation is important to the future development of therapeutic approaches to neuronal injuries and neurodegenerative diseases. Recent studies have shown that environmental signals such as various cytokines and growth factors influence proliferation or differentiation of NSCs (Ahmed et al., 1995; Tropepe et al., 1997, 1999; Arsenijevic and Weiss, 1998; Qian et al., 2000). The present study was conducted to determine the effects of HGF on NSCs in vitro.

HGF was first identified as a potent mitogen for hepatocytes, and later purified and molecularly cloned in 1989 (Nakamura et al., 1989). HGF has various effects not only on hepatocytes but also on various types of cells. Recent extensive analyses of HGF have revealed that HGF is a pleiotrophic factor which induces a variety of responses in normal development and in pathological situations (Matsumoto and Nakamura, 1997). HGF might also play a role during the early steps of neuronal induction. The expression of both HGF and c-Met, an HGF receptor expressed during brain development, persists in the adult (Jung et al., 1994; Achim et al., 1997; Streit et al., 1997, Maina and Klein, 1999). Accordingly, it has been reported that HGF has a variety of effects such as antiapoptosis, morphogenesis, motility, and mitogenesis of neural cells (Honda et al., 1995; Hamanoue et al., 1996; Ebens et al., 1996; Novak et al., 2000).

In the present study, immunoreactivity of c-Met receptor was observed on cells in neurospheres isolated from E14 mouse embryos, suggesting that HGF has its receptor on the cells in neurospheres. Without FGF-2 or EGF, neurosphere formation was observed with the medium containing only HGF, although size and the number of cells obtained with HGF were less than with FGF-2, EGF, or their combination. Neurospheres formed with HGF contained immunopositive cells for nestin and were multipotent, yielding neurons, astrocytes, and oligodendrocytes. However, the ability to form neurospheres was reduced after repeated subculture by mechanical dissociation of the neurospheres. This fact suggests that the neurospheres formed with only HGF are not derived from NSCs but are neural progenitors. Accordingly, HGF may promote production of neural progenitors rather than division of NSCs. The increase in number and size of primary neurospheres can be also explained by an antiapoptotic effect on NSCs of HGF. It is known that neurospheres contain both stem cells and neuronal/glial progenitor cells already committed to neuron/glia (Reynolds and Weiss, 1996; Svendsen and Caldwell, 2000).

There are two proliferative divisions in NSCs: symmetric division to produce neural stem cells, and asymmetric division to produce progenitor cells. It is also known that the neurogenic phase producing mainly neuronal progenitors is early, and the gliogenic phase producing glial progenitors is late (Morrison et al., 1997; Qian et al., 2000). It is likely that neurospheres formed by HGF contain predominantly progenitor cells committed to neurons or glia. We cannot discriminate these progenitor cells from NSCs because they are also immunopositive for nestin. In other words, HGF promotes asymmetric division rather than symmetric division. These hypotheses may explain the reduced ability for self-renewal of the cells isolated in the medium containing HGF.

In the present study, we obtained interesting findings whereby the addition of HGF to medium containing FGF-2, EGF, or their combination increased the number and size of neurospheres in primary culture. On the other hand, when the cells in the neurospheres were dissociated and cultured with FGF+EGF, the number of neurospheres formed was smaller when generated by HGF or FGF+EGF+HGF than by FGF+EGF. This phenomenon may be explained by the hypotheses that (1) HGF promotes proliferation and differentiation of neural progenitors, and (2) HGF inhibits apoptosis or necrosis of neural progenitors.

In support of hypothesis 1, addition of HGF to growth medium containing FGF-2, EGF, or their combination increased the number of BrdU-positive cells as shown in this study. It was also suggested that HGF increased the number and size of neurospheres and that most of the cells in these neurospheres generated with HGF were neural progenitors, leading to limited self-renewal ability. Furthermore, more neurons were obtained after culture in growth medium containing HGF. These results suggest that HGF promoted asymmetric division, producing more neuronal progenitor cells than glial progenitor cells. Next, with respect to hypothesis 2, it has been reported that a significant amount of cell death occurs during CNS development (Oppenheim, 1991). Interestingly, most of the TUNEL-positive cells are located in the periventricular zone (PVZ) where NSCs exist in the brain (Thomaidou et al., 1997; Blaschke et al., 1996). It is also known that cell death is occurring at the center of neurospheres during both growth and differentiation (Erlandsson et al., 2001). Apoptosis seemed to be closely involved in these cell deaths. Our results have also shown that there are many TUNEL-positive cells in neurospheres cultured in growth medium. The antiapoptotic effect of HGF might lead to an increase in both the number and size of neurospheres in addition to the proliferative effects of neural progenitors as mentioned above. We could not elucidate whether the addition of HGF had an antiapoptotic effect on neuronal progenitors or glial progenitors despite our efforts with additional experiments using double staining for TUNEL and neuronal/glial markers in neurospheres. Transcription factors that regulate maintenance and differentiation of NSCs have been extensively investigated, and it was reported that activator-type basic helix–loop–helix, Mash1, Math1, Math3, and Neurogenin2 promote neurogenesis (Tomita et al., 2000; Nieto et al., 2001). It remains unknown whether these signaling pathways are involved in the proliferative and differentiating effects of HGF on NSCs.

Thus, HGF promotes proliferation and neuronal differentiation of NSCs isolated from E14 mouse embryos. Further understanding of the mechanisms underlying the effects of HGF on NSCs may lead to the development of new biological techniques or treatments to control and regulate NSCs from brains to be used as novel therapeutic approaches for neuronal injuries and disorders.

Experimental methods

Primary culture and neurosphere passage

Striatal cells were removed from 14-day-old mouse embryos (C57BL/6, plug day = 1.0) in PBS buffer containing penicillin (50 U/ml) and streptomycin (50 U/ml) (both from ICN Pharmaceuticals). The tissue was mechanically dissociated with a fire-polished pipet in serum-free medium consisting of DMEM and F-12 nutrient (1:1; Invitrogen). The cells were grown in growth medium in Falcon culture flasks (Falcon) or 6-well dishes (Falcon) or 24-well dishes (Falcon) at a concentration of 150,000 cells/ml. The growth medium contained DMEM and F-12 nutrient (1:1; Invitrogen), glucose (0.6%), glutamine (2 mM), B27 supplement (2%, Invitrogen), and EGF, FGF-2, and/or HGF (R&D Systems) at a concentration of 20 ng/ml each. Half of the medium was changed every 4 days with fresh medium containing the same concentrations of growth factors. After 7 days, primary neurospheres were collected by centrifugation (2300 *g*), resuspended in fresh medium, and dissociated with a fire-polished pipet as described above.

To assess the effects of HGF on the size and number of neurospheres in primary culture, E14 striatal cells were cultured for 7 days in growth medium containing FGF-2 (20 ng/ml) and/or EGF (20 ng/ml), with various concentrations of added HGF. The primary neurospheres were counted and under a phase-contrast microscope (IMT-2, Olympus, Japan).

Secondary neurosphere formation assay

Primary neurospheres were collected and digested with 0.25% trypsin–EDTA (Sigma–Aldrich) for 5 min at 37°C. They were then gently triturated with a fire-polished pipet, spun down at 500 rpm for 3 min, resuspended in growth medium, and plated at 500 cells/200 μl in each well of a 96-well dish (Falcon). After 7 days, the secondary neurospheres were counted.