ACADEMIC PRESS

Biochemical and Biophysical Research Communications 293 (2002) 167–173

BBRC

www.academicpress.com

Wnt-1 promotes neuronal differentiation and inhibits gliogenesis in P19 cells

K. Tang,[a] J. Yang,[a,1] X. Gao,[a] C. Wang,[a] L. Liu,[a] H. Kitani,[b] T. Atsumi,[c] and N. Jing[a,*]

[a] *Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China*

[b] *Department of Molecular Biology and Immunology, National Institute of Agrobiological Sciences (NIAS), 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan*

[c] *The Laboratory of Molecular Cell Science, Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan*

Received 15 March 2002

Abstract

Wnt-1, the vertebrate counterpart of the *Drosophila wingless* gene, plays an important role in the early morphogenesis of neural tissues. In this report, we have shown that overexpression of *Wnt-1* can direct embryonic carcinoma P19 cells to differentiate into neuron-like cells in the absence of retinoic acid. Immunocytochemistry showed that these cells expressed neuronal markers, such as the neurofilament (NF) and microtubule-associated protein 2 (MAP2), but failed to express the glial cell marker, glial fibrillary acidic protein (GFAP). RT-PCR revealed that two basic helix-loop-helix (bHLH) genes, *Mash-1* and *Ngn-1*, were up-regulated during the differentiation stage of Wnt-1-overexpressing P19 cells. These results suggest that the *Wnt-1* gene promotes neuronal differentiation and inhibits gliogenesis during the neural differentiation of P19 cells, and that neural bHLH genes might be involved in this process.

Keywords: *Wnt-1*; P19 embryonic carcinoma cell; Neural differentiation; Neuronal; Glial; bHLH genes; *Mash-1*; *Ngn-1*

Neurogenesis is one of the most complex and hierarchical events in the development of vertebrates. Several classes of secreted factors, among which are FGF, Wnt, Hedgehog, and TGFβ families, have been speculated to be candidate signals that specify the fate of neural cells [1–3]. Wnt genes, encoding a large family of secreted, cysteine-rich proteins, are implicated in a wide variety of biological processes, such as embryonic patterning, cell proliferation, and cell fate determination [4,5]. *Wnt-1*, the vertebrate counterpart of the *Drosophila wingless* gene [6], is expressed mainly in the developing central nervous system (CNS) during vertebrate embryogenesis [7,8]. Targeted disruption of the *Wnt-1* gene in mice results in loss of both midbrain and cerebellar structures [9,10]. It was shown recently that Wnt signaling in *Xenopus* embryos can activate neural development [11]. These observations suggest that the *Wnt-1* gene may function as a neural determination factor in the morphogenesis of the neural tube and/or the early stages of CNS development.

The P19 cell is a mouse embryonic carcinoma cell line, which could be induced by retinoic acid (RA) to differentiate into neuroectodermal cell lineages, such as neurons and glial cells [12–14]. It was revealed that the *Wnt-1* gene expression was induced at mRNA and protein levels after RA induction in P19 cells [15–17]. Smolich and Papkoff [18] reported that overexpression of *Wnt-1* in P19 cells could activate expression of other Wnt genes, but these cells could not differentiate into neuron-like cells without RA. However, we showed that overexpression of *N-cadherin* could direct P19 cells to differentiate into neuronal cells in the absence of RA possibly through the Wnt-1 signaling pathway [19], and

* Corresponding author. Fax: +86-21-64338357.
E-mail address: njing@server.shcnc.ac.cn (N. Jing).

[1] Present address: Howard Hughes Medical Institute and Department of Medicine, University of Pennsylvania School of Medicine.

0006-291X/02/$ - see front matter
PII: S0006-291X(02)00215-2

also the aggregates of *Wnt-1*-overexpressing P19 (Wnt-1/P19) cells could differentiate into neural cells without RA induction [20]. In this report, we show that Wnt-1/P19 cells, when replated as the single cell suspension, could differentiate only into neurons in the absence of RA, while the gliogenesis was inhibited. We further explored the molecular mechanisms underlying Wnt-1/P19 cell neural differentiation, and found that the neural bHLH genes might be involved in this process.

Materials and methods

P19 cell culture and RA-induced neuronal differentiation. P19C6, a subclone of the P19 mouse embryonic carcinoma cell line, was used in this study [19]. P19C6 cells were cultured as previously described [21]. To induce neural differentiation, P19 cells were allowed to aggregate in bacterial grade petri dishes (Fisher) at a seeding density of 1×10^5 cells/ml in the presence of 1×10^{-6} M all-*trans*-retinoic acid (RA, Sigma) in 10% fetal bovine serum (FBS)/αMEM (Gibco). After 4 days of aggregation, cells were dissociated into single cells by 0.05% trypsin-0.53 mM EDTA (Gibco), and were replated in a poly-L-lysine coated tissue culture dish at a density of 1×10^5 cells/cm^2 in a N2 serum-free medium (DMEM/F12 supplemented with 5 μg/ml insulin, 50 μg/ml human transferrin, 20 nM progesterone, 60 μM putresine, and 30 nM sodium selenite) supplemented with 1 μg/ml fibronectin (Gibco). The cells were then allowed to adhere and cultured for 2–3 weeks with replacement of the medium every 48 h. The same protocol was used for Wnt-1/P19 cells except for the absence of RA during the aggregation stage.

RT-PCR. Total RNA was prepared from cells using Trizol reagent (Gibco). Reverse transcription was performed with 5 μg of total RNA using SuperScript II reverse transcriptase (Gibco). PCR amplification was carried out with a reverse transcribed template DNA 50 ng, primers 10 pmol, dNTP 0.2 mM, and α-^{32}P-dATP 10 nCi. PCR reaction consisted of denaturation at 94 °C for 45 s, annealing for 45 s, and extension at 72 °C for 1 min. PCR primers and reaction parameters for each gene are shown in Table 1. PCR products were electrophoresed on 5% polyacrylamide gels, air dried, and processed for autoradiography. PCR bands were analyzed by Gelworks 1D Advanced computer software (UVP). In the non-radioisotope PCR, the products were electrophoresed on 1.2% agarose gels containing ethidium bromide. Reverse transcription-minus RNA samples were used as negative controls for each pair of primers, and no positive band was detected. PCR analyses were repeated 3–4 times for each gene, and similar results were obtained.

Immunofluorescence staining. Immunocytochemistry was performed as previously described [22]. Mouse monoclonal antibody against microtubule-associated protein 2 (MAP2) and rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP) were purchased from Sigma (USA), and mouse monoclonal antibody 160 kDa neurofilament (NF160) was purchased from Roche (Germany). Both secondary antibodies, DTAF-conjugated goat-anti-mouse IgG and CY3-conjugated goat-anti-rabbit IgG, were obtained from Jackson ImmunoResearch Laboratories (Pennsylvania, USA). Normal mouse IgG (Zymed) was used as the negative control.

Results

Wnt-1 directs neural differentiation of P19 cells

Our previous study suggested that Wnt-1/P19 cells, when replated as the aggregates, could differentiate into neural cells without RA induction [20]. However, RA-induced P19 cells were normally replated as the single cell suspension to enrich the cells of interest during their neural differentiation [21,23]. To further confirm whether *Wnt-1* overexpression could initiate P19 cell neural differentiation as RA-treatment, two different subclones of Wnt-1/P19 cell (Wnt-1/P19-A, Wnt-1/P19-T) were aggregated for 4 days in the absence of RA, then replated as the single cell suspension and allowed to differentiate for three weeks. The wild-type P19 cells as well as the neomycin resistant empty expression vector-transfected P19 cell subclone (Neo$^+$/P19) were used as the negative controls. As shown in Fig. 1A, *Wnt-1*-overexpressing P19 cells (clone Wnt-1/P19-T) had a polygonal epithelial cell-like morphology similar to the control wild-type P19 and Neo$^+$/P19 cells at day 1 after replating. By day 2, the cytoplasmic volume of Wnt-1/P19-T cells started to decrease, and the cells showed bipolar morphology with short processes (data not shown). At day 4, Wnt-1/P19-T cells started to send out long neurite-like processes and began to form small cell clusters (Fig. 1B). By day 7, the cell clumps were getting bigger, and more neurite-like processes appeared (Fig. 1C). At day 10 after replating, the Wnt-1/P19-T cell clumps sent out many neurite-like fibers to form the networks (Fig. 1D). By day 14, Wnt-1/P19-T cells formed the most abundant neurite-like networks (Fig. 1E). At day 19, the neurite-like networks started to degenerate (Fig. 1F). The RA-induced wild-type P19 cells

Table 1
PCR primers and reaction parameters for different genes

	5′ Primer	3′ Primer	Annealing temperature (°C)	Cycles	Size (bp)
GFAP	tttctccttgtctcgaatga	ggtttcatcttggagcttct	55	30	370
En-2	agtcccgaccgaacccagcg	gtccgagtcggagctcaccg	63	29	212
Hoxa-7	tctatcagagccccttcgcgt	tgcctggccctttactcctctt	55	33	520
Oct-3/4	cagaagaggatcaccttggg	gtgagtgatctgctgtaggg	60	27	324
BMP-4	tgccgcagcttctctgagcc	gctctgccgaggagatcacc	64	26	573
Mash-1	caagttggtcaacctgggtt	gctcttgttcctctgggcta	56	30	293
Ngn-1	cctttggagacctgcatctc	gatgtagttgtaggcgaagc	52	28	417
β-Actin	tcgtcgacaacggctccggcatgt	ccagccaggtccagacgcaggat	56	19	520

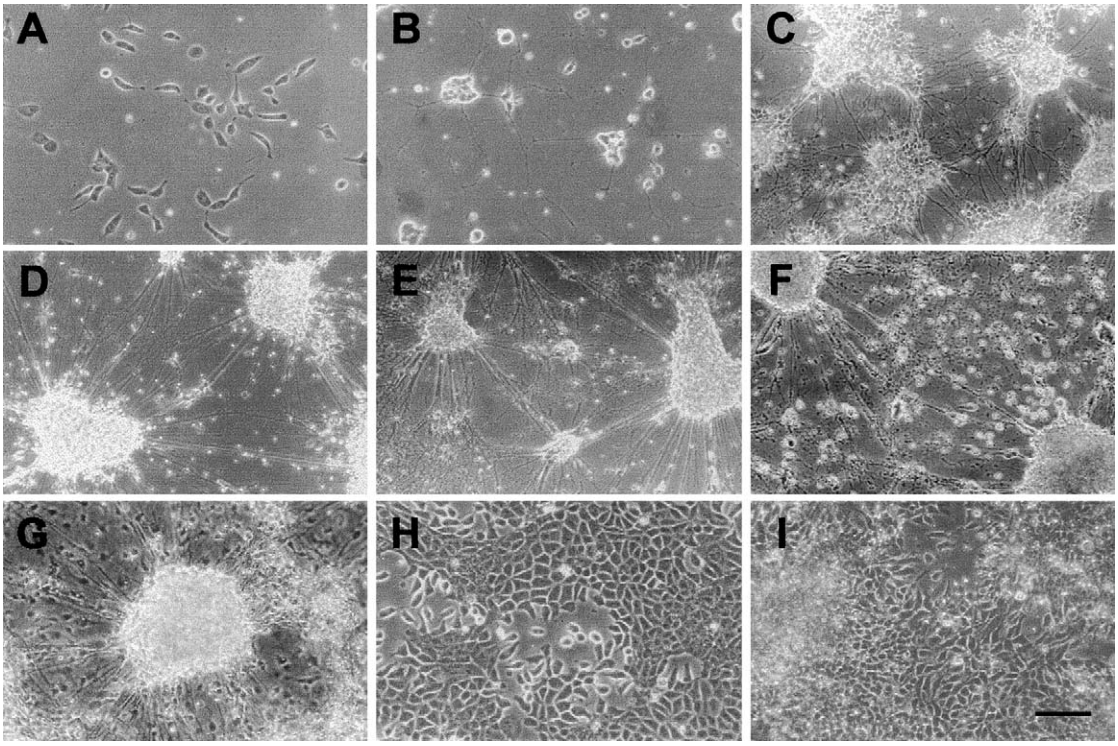

Fig. 1. Morphology of Wnt-1/P19 cells during their neural differentiation. Wnt-1/P19 cells were aggregated for 4 days in the absence of RA, then replated as the single cell suspension, and cultured in N2 medium for 1 day (A), 4 days (B), 7 days (C), 10 days (D), 14 days (E), and 19 days (F), respectively. RA-induced wild-type P19 cells were replated as the single cell suspension in N2 medium for 10 days (G). Control wild-type P19 cells and Neo$^+$/P19 cells were aggregated without RA for 4 days, and replated similarly in N2 medium for 2 days for P19 cells (H) and for 7 days for Neo$^+$/P19 cells (I), respectively. Note that Wnt-1/P19 cells formed long neurite-like processes after aggregation without RA induction, while wild-type P19 cells and Neo$^+$/P19 cells retained typical epithelial cell-like morphology. In the culture of RA/P19 cells, underlying the cell clusters there was a layer of cells among which some cells had the astrocyte-like morphology (G). Scale bar, 50 μm.

(RA/P19), after being replated as the single cell suspension, could also form the big cell clusters with many neurite-like processes. Underlying the cell clusters, there was a layer of cells among which some cells had the astrocyte-like morphology (Fig. 1G). In contrast, we could not observe any glia-like cells in the cultures of Wnt-1/P19 cell even after being replated for 2–3 weeks (Fig. 1E and F). The control wide-type P19 cells and Neo$^+$/P19 cells continued to proliferate with no morphological changes, after being replated as the single cell suspension without RA induction (Fig. 1H and I).

Immunofluorescence staining was used to verify the neuronal marker expression in neuron-like cells derived from Wnt-1/P19-T cells. Wnt-1/P19-T cells started to express neuronal markers, MAP2 and NF160, as early as day 3 after replating (data not shown). At day 9 after replating, both the cell bodies and neurite-like processes of Wnt-1/P19-T cells were strongly positive with anti-MAP2 and anti-NF160 antibodies (Fig. 2A and B). However, Neo$^+$/P19 cells were negative for both MAP2 and NF160 antibodies in the same culture conditions (Fig. 2C and D). Two clones, Wnt-1/P19-A (data not shown) and Wnt-1/P19-T (data shown), differentiated in the same manner.

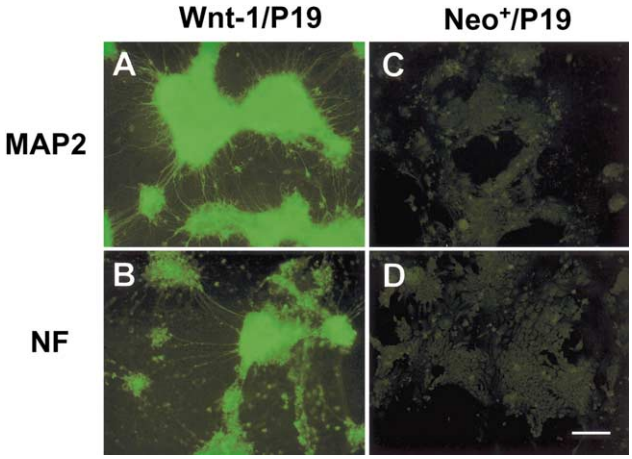

Fig. 2. Immunocytochemical characterization of neuron-like cells differentiated from Wnt-1/P19 cells. After 4 days aggregation in the absence of RA, Wnt-1/P19 and Neo$^+$/P19 cells were dissociated, and replated in N2 medium for 9 days. The cells were stained with anti-MAP2 (A, C) and anti-NF160 (B, D) antibodies, respectively. Note that Wnt-1/P19 cells formed long, fine neurite-like processes, and were strongly positive for both MAP2 (A) and NF160 (B). While Neo$^+$/P19 cells were negative for both MAP2 (C) and NF160 (D) antibodies. Scale bar, 100 μm.

Together with our previous report [20], these results suggest that the forced expression of *Wnt-1* bypasses the requirement for RA to initiate neural differentiation in P19 cells, and that the *Wnt-1* gene might be one of the important downstream targets of RA signaling in P19 cell neural differentiation.

Overexpression of the Wnt-1 gene promotes P19 cell neuronal differentiation and inhibits gliogenesis

Since the cultures derived from the RA/P19 cells contain both neurons and glial cells [12–14], we also examined whether and when glial cells start to differentiate during the Wnt-1/P19 cell neural differentiation. Consistent with the morphological observation, double staining showed that there was no glial fibrillary acidic protein (GFAP, a glial cell marker)-positive cell in the culture of Wnt-1/P19 cells, even if many cells were strongly positive for NF160 by day 19 after replating (Fig. 3A, right column). In contrast, GFAP-positive cells started to appear in the culture of RA/P19 cells at day 9 after replating (data not shown), and many cells with the classical astrocytic morphology were positive with GFAP but negative for NF160 at day 13 (Fig. 3A, left column). Accordingly, GFAP mRNA could readily be found in RA/P19 cells at day 9 and 14 by RT-PCR analysis. On the contrary, we could not detect the expression of GFAP in the Wnt-1/P19 derived neural cells as late as day 14 after replating (Fig. 3B). These results demonstrate that Wnt-1/P19 cells only differentiate into neurons, and strongly suggest that overexpression of the *Wnt-1* gene promotes P19 cell neuronal differentiation and inhibits gliogenesis.

The CNS regional identity of neural cells derived from RA/P19 and Wnt-1/P19 cells

In order to determine whether *Wnt-1* expression was interfering with the regional identity of differentiating *Wnt-1*/P19 cells, we selected three CNS region-specific genes to analyze and compare their expression patterns during the neural differentiation of RA/P19 and Wnt-1/P19 cells. *Otx-1* [24], *En-2* [25], and *Hoxa-7* [26] are expressed in the forebrain, the midbrain and rostral hindbrain, and the posterior spinal cord, respectively. We could not detect *Otx-1* expression in either RA/P19 cells or *Wnt-1*/P19 cells (data not shown). However, the expression of *En-2* and *Hoxa-7* was up-regulated during neural differentiation of these cells, and the expression profiles of these genes were similar between RA/P19 cells and Wnt-1/P19 cells (Fig. 3C). During neurogenesis in vivo, RA is important for the patterning of the hindbrain [27], and *Wnt-1* gene is also a key regulator of met-mesencephalic development [28]. These results are consistent with the roles of RA and *Wnt-1* in CNS development in vivo, and suggest that neural cells derived from RA/P19 and Wnt-1/P19 cells have a phenotype similar to that of midbrain and hindbrain cells in vivo.

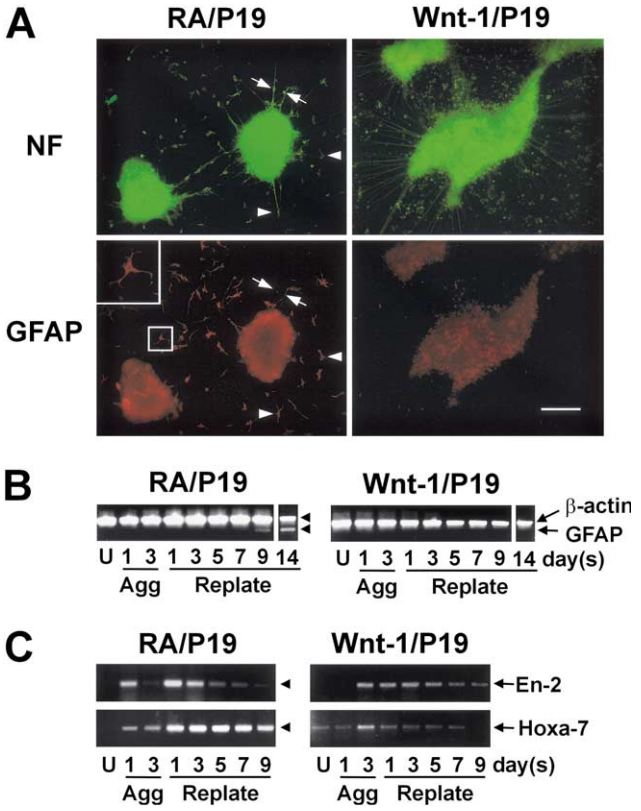

Fig. 3. The expression of neuronal and glial cell markers during Wnt-1/P19 cell neural differentiation and expression of CNS regional-specific genes. (A) Wnt-1/P19 cells express the neuronal marker but not the glial cell marker. RA/P19 and Wnt-1/P19 cells were replated in N2 medium for 13 and 19 days, respectively. The cells were double-stained with anti-NF160 and anti-GFAP antibodies. In RA/P19 cells (left column), there were many cells strongly positive with NF160, but negative with GFAP (arrows); there were also GFAP-positive and NF160-negative cells (arrowheads) with typical astrocyte morphology (inset). In Wnt-1/P19 cells (right column), however, there were only NF160-positive cells, and no GFAP-positive cells. Scale bar, 100 μm. (B) RT-PCR analysis of GFAP mRNA expression during neural differentiation of RA/P19 and Wnt-1/P19 cells. The primers for β-actin (3 pmol) were added together with the primers for GFAP (10 pmol) as the internal control. For RT-PCR analysis, samples were prepared from cell lysates of undifferentiated cells (lane U), cells aggregated for 1 day (lane Agg-day 1) and 3 days (lane Agg-day 3), and from cells replated in N2 medium for 1, 3, 5, 7, 9, and 14 days, respectively (lanes Replate-day 1, 3, 5, 7, 9, and 14). RT-PCR products were electrophoresed on 1.2% agarose gels containing ethidium bromide. Note that the GFAP transcripts could be detected in RA/P19 cells at day 9 and 14, while there is no GFAP expression in Wnt-1/P19 cells. (C) RT-PCR analysis of En-2, Hoxa-7 mRNA expression during neural differentiation of RA/P19 and Wnt-1/P19 cells. All experiments were repeated 3–4 times, and similar results were obtained.

Expression profiles of regulatory genes during Wnt-1/P19 cell neural differentiation

The previous data suggest that overexpression of the *Wnt-1* gene triggered neuronal but not glial differentia-

tion of P19 cells. To further explore the molecular mechanisms underlying *Wnt-1*-induced neuronal differentiation, RT-PCR was used to examine the expression patterns of those developmental regulatory genes, such as *Oct-3/4* [29], BMP-4 [30], *Mash-1* [31], and *Ngn-1* [32] in RA/P19 and Wnt-1/P19 cells (Fig. 4). Among these genes, expression patterns of *Oct-3/4* and BMP-4 were, in general, similar between these cells, although *Oct-3/4* had a slower decreasing rate and BMP-4 was expressed in undifferentiated Wnt-1/P19 cells (Fig. 4A and B). *Mash-1* was not expressed in both wild-type P19 cells and Wnt-1/P19 cells in their undifferentiated state and during their induction/aggregation stage. In RA/P19 cells, *Mash-1* expression started at day 1 after replating, reached its peak by day 3, then it was down-regulated and maintained later on at a low level. Similarly, in Wnt-1/P19 cells *Mash-1* expression began also at day 1 after replating, but continued increasing until day 9 (Fig. 4A and B). *Ngn-1* expression was induced by RA at day 1 of aggregation in wild-type P19 cells, and kept at a relatively high level until day 9 after replating. In Wnt-1/P19 cells, however, *Ngn-1* expression started only at day 3 after replating, and was up-regulated continuously until day 9 (Fig. 4A and B). Together, these results show that in Wnt-1/P19 cells the bHLH genes, such as, *Mash-1* and *Ngn-1*, begin to be expressed only in the neural differentiation stage, and suggest that these neural bHLH genes might be involved in the neural differentiation of Wnt-1/P19 cells.

Discussion

In this study, we have further addressed the function of the *Wnt-1* gene in the neural differentiation of P19 cells. Our studies reveal that overexpression of *Wnt-1*

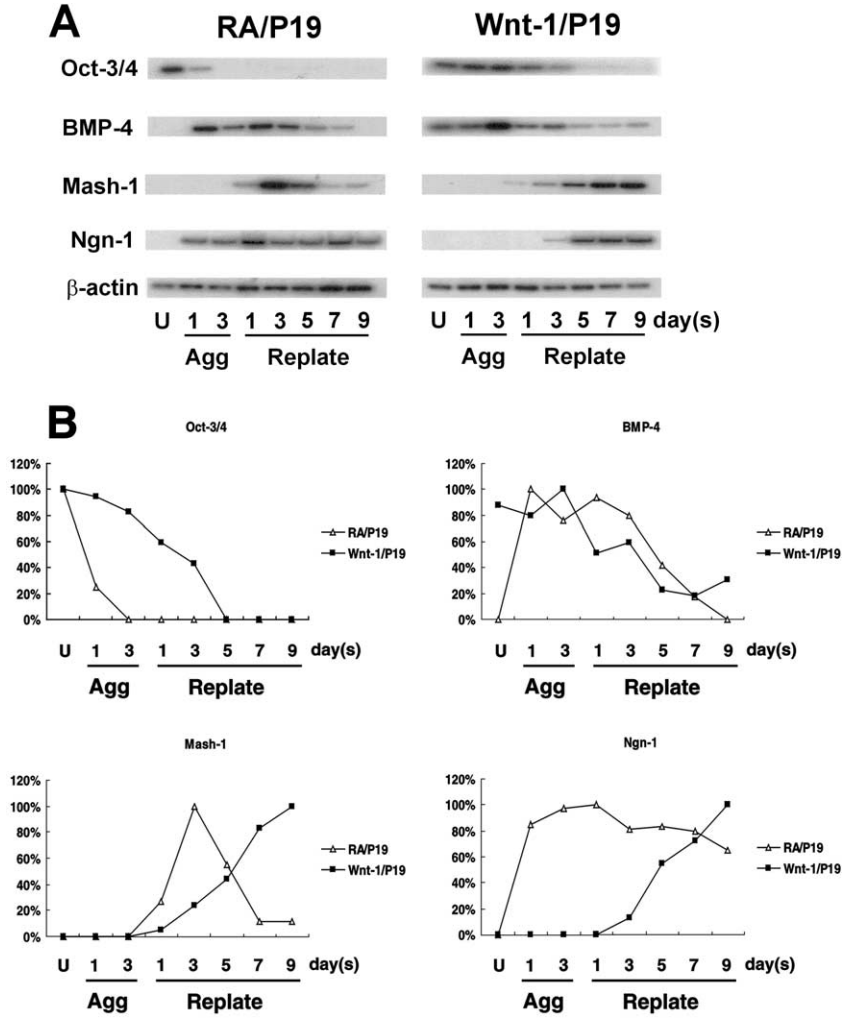

Fig. 4. The expression patterns of regulatory genes during neural differentiation of RA/P19 and Wnt-1/P19 cells. (A) RT-PCR analysis of *Oct-3/4*, BMP-4, *Mash-1*, and *Ngn-1* mRNA expression in differentiating RA/P19 and Wnt-1/P19 cells. Samples were prepared from cell lysates of undifferentiated cells (lane U), cells aggregated for 1 day (lane Agg-day 1) and 3 days (lane Agg-day 3), and cells replated in N2 medium for 1, 3, 5, 7, and 9 days, respectively (lanes Replate-day 1, 3, 5, 7, and 9). (B) Semiquantitative analysis of expression of regulatory genes during neural differentiation of RA/P19 and Wnt-1/P19 cells (–△– RA/P19, –■– Wnt-1/P19). All experiments were repeated 3–4 times, and similar results were obtained.

can direct neuronal differentiation of P19 cells in the absence of RA, when Wnt-1/P19 cells were replated as the single cell suspension. Furthermore, we show that *Wnt-1*-overexpressing P19 cells differentiate exclusively into neurons but not glial cells. By examining the expression pattern of several regulatory genes, we provide evidence that *Mash-1* and *Ngn-1* are potentially involved in the neural differentiation of Wnt-1/P19 cells.

Chemical inducers and aggregation are two key elements to impart fate choices of P19 cells. With aggregation, DMSO directs P19 cells to differentiate into mesoderm origin muscle cells. RA, however, induces P19 cells to differentiate into ectoderm-derived neural cells including neurons and astrocytes [12–14]. According to the treatment of cells, the neural differentiation of RA-induced P19 cells can be divided into two sequential stages, a stage of induction and a stage of differentiation. During the first stage, P19 cells are allowed to aggregate in the petri dish and induced with RA for four days. Based on cell morphology changes and gene expression profiles, it seems that pluripotent P19 embryonic carcinoma (EC) cells are determined into neural progenitor cells during the first RA-induction stage. In the second stage, the induced P19 cells are replated into cell culture dish as the single cell suspension or aggregates and left to differentiate into mature neurons and astrocytes.

Smolich and Papkof [18] reported that ectopic expression of *Wnt-1* was capable of initiating differentiation of P19 cells by inducing *Wnt-4* and *Wnt-6* expressions, but it alone was not sufficient to induce terminally differentiated neuronal phenotypes. Our results, however, demonstrated that overexpression of the *Wnt-1* gene itself was sufficient not only to commit P19 cells to a neural fate, but also to promote the terminal differentiation of these cells into mature neurons. The discrepancy can be explained either by differences in the original P19 cell subclones that the two groups have used or by the different induction protocols for *Wnt-1*-overexpressing cells used in the two laboratories. We favor the second explanation, since in our experiments, *Wnt-1* /P19 cells were aggregated in absence of RA for four days, whereas *Wnt-1*-overexpressing P19 cells were treated as a monolayer without RA in their report [18]. Aggregation is important for the neural fate determination of P19 cells, and it may provide a cellular environment necessary for efficient Wnt-1 activity. Presumably, *Wnt-1* overexpression can only partially substitute the function of RA and the cell aggregation is still needed to initiate the neuronal differentiation of P19 cells.

As the neurogenesis in vivo, neurons appear earlier than glial cells during RA-induced P19 cell neural differentiation [12]. Interestingly, our results show that Wnt-1/P19 cells differentiate restrictively into neurons, but not glial cells (Fig. 3). In parallel, the expression of two neural bHLH genes, *Mash-1* and *Ngn-1*, is up-regulated during the differentiation stage of Wnt-1/P19 cells (Fig. 4). This suggests that *Wnt-1* may promote the neuronal fate and inhibit glial fate in P19 cells through activation of neural bHLH gene expression. Neural bHLH genes have recently been shown, in addition to their neural determination function, to play important roles in the neuronal versus glial fate decision. In *Mash-1* and *Math-3* double mutant mice, generation of neurons is blocked at the neural precursor stage in the regions where the two genes are coexpressed, and instead, premature astrocytic differentiation is observed in the same regions [33]. When *Mash-1* and *Ngn-2* are both inactivated in mice, progenitors of the cerebral cortex differentiate prematurely and excessively into astrocytes instead of neurons [34]. *Ngn-1* has been shown to promote neurogenesis by functioning as a transcriptional activator, while inhibiting astrocyte differentiation by sequestering the CBP-Smad transcription complex away from astrocyte differentiation genes and inhibiting the activation of STAT transcription factors [35]. Furthermore, the cotransfection of E12 and *NeuroD2*, *Mash-1*, *Ngn-1*, or other bHLH genes is sufficient to convert P19 cells into differentiated neurons in the absence of RA [36]. In *Drosophila*, the expression of *achaete* can be induced by *wingless* in wing imaginal disc [37,38]. Thus, it is possible that as in *Drosophila*, overexpression of *Wnt-1* induces the expression of neural bHLH genes, that in turn promotes neuronal differentiation and inhibits gliogenesis during the neural differentiation of P19 cells. Further experiments will be needed to provide the direct evidence for the crosstalk between the Wnt signaling pathway and bHLH genes.

Acknowledgments

We thank Dr. Francois Guillemot and Steve Kulich for the critical reading of the manuscript. This work was supported in part by grants from National Basic Research Program (G1999054000) and National Natural Science Foundation of China (39870283, 39930090).

References

[1] L. Lillien, Neural progenitors and stem cells: mechanisms of progenitor heterogeneity, Curr. Opin. Neurobiol. 8 (1998) 37–44.
[2] J.L.R. Rubenstein, P.A. Beachy, Patterning of the embryonic forebrain, Curr. Opin. Neurobiol. 8 (1998) 18–26.
[3] K.J. Lee, T.M. Jessell, The specification of dorsal cell fates in the vertebrate central nervous system, Annu. Rev. Neurosci. 22 (1999) 261–294.
[4] A. Wodarz, R. Nusse, Mechanisms of Wnt signaling in development, Annu. Rev. Cell Dev. Biol. 14 (1998) 59–88.
[5] A. Patapoutian, L.F. Reichardt, Role of Wnt proteins in neural development and maintenance, Curr. Opin. Neurobiol. 10 (2000) 392–399.
[6] F. Rijsewijk, M. Schuermann, E. Wagenaar, P. Parren, D. Weigel, R. Nusse, The *Drosophila* homolog of the mouse mammary

oncogene *int-1* is identical to the segment polarity gene *wingless*, Cell 50 (1987) 649–657.
[7] R. Nusse, H.E. Varmus, Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome, Cell 31 (1982) 99–109.
[8] A.P. McMahon, A.L. Joyner, A. Bradley, J.A. McMahon, The midbrain–hindbrain phenotype of *Wnt*-1^{-}/*Wnt*-1^{-} mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum, Cell 69 (1992) 581–595.
[9] A.P. McMahon, A. Bradley, The *Wnt-1* (*int-1*) proto-oncogene is required for development of a large region of the mouse brain, Cell 62 (1990) 1073–1085.
[10] K.R. Thomas, M.R. Capecchi, Targeted disruption of the murine *int-1* proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development, Nature 346 (1990) 847–850.
[11] J.C. Baker, R.S.P. Beddington, R.M. Harland, Wnt signaling in *Xenopus* embryos inhibits bmp-4 expression and activates neural development, Genes Dev. 13 (1999) 3149–3159.
[12] E.M. Jones-Villeneuve, M.W. McBurney, K.A. Rogers, V.I. Kalnins, Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells, J. Cell Biol. 94 (1982) 253–262.
[13] E.M. Jones-Villeneuve, M.A. Rudnicki, J.F. Harris, M.W. McBurney, Retinoic acid-induced neural differentiation of embryonal carcinoma cells, Mol. Cell. Biol. 3 (1983) 2271–2279.
[14] M.W. McBurney, E.M. Jones-Villeneuve, M.K. Edwards, P.J. Anderson, Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line, Nature 299 (1982) 165–167.
[15] R. St.-Arnaud, J. Craig, M.W. McBurney, J. Papkoff, The *int-1* proto-oncogene is transcriptionally activated during neuroectodermal differentiation of P19 mouse embryonal carcinoma cells, Oncogene 4 (1989) 1077–1080.
[16] E. Schuuring, L. van Deemter, H. Roelink, R. Nusse, Transient expression of the proto-oncogene *int-1* during differentiation of P19 embryonal carcinoma cells, Mol. Cell. Biol. 9 (1989) 1357–1361.
[17] J. Papkoff, Identification and biochemical characterization of secreted Wnt-1 protein from P19 embryonal carcinoma cells induced to differentiate along the neuroectodermal lineage, Oncogene 9 (1994) 313–317.
[18] B.D. Smolich, J. Papkoff, Regulated expression of Wnt family members during neuroectodermal differentiation of P19 embryonal carcinoma cell: overexpression of *Wnt-1* perturbs normal differentiation-specific properties, Dev. Biol. 166 (1994) 300–310.
[19] X. Gao, W. Bian, J. Yang, K. Tang, H. Kitani, T. Atsumi, N. Jing, A role of *N*-cadherin in neuronal differentiation of embryonic carcinoma P19 cells, Biochem. Biophys. Res. Commun. 284 (2001) 1098–1103.
[20] J. Yang, H. Sun, W. Bian, N.H. Jing, Neural differentiation of Wnt-1 overexpression P19 cells, Sheng Li Xue Bao 50 (1998) 289–295.
[21] M.A. Rudnicki, M.W. McBurney, Cell culture methods and induction of differentiation of embryonal carcinoma cell lines, in: E.J. Robertson (Ed.), Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, IRL Press, Washington DC, 1987, pp. 19–49.
[22] N.H. Jing, R. Shiurba, H. Kitani, H. Kawakatsu, Y. Tomooka, T. Sakakura, Secretion of polypeptides related to epidermal growth factor and insulinlike growth factor I by a human teratocarcinoma cell line, In Vitro Cell Dev. Biol. 27A (11) (1991) 864–872.
[23] P.A. MacPherson, M.W. McBurney, P19 embryonal carcinoma cells: a source of cultured neurons amenable to genetic manipulation, Methods 7 (1995) 238–252.
[24] A. Simeone, D. Acampora, M. Gulisano, A. Stornaiuolo, E. Boncinelli, Nested expression domains of four homeobox genes in developing rostral brain, Nature 358 (1992) 687–690.
[25] A.L. Joyner, G.R. Martin, *En-1* and *En-2*, two mouse genes with sequence homology to the *Drosophila* engrailed gene: expression during embryogenesis, Genes Dev. 1 (1987) 29–38.
[26] K.A. Mahon, H. Westphal, P. Gruss, Expression of homeobox gene Hox 1.1 during mouse embryogenesis, Development 104 (Suppl.) (1988) 187–195.
[27] S. Guthrie, Patterning the hindbrain, Curr. Opin. Neurobiol. 6 (1996) 41–48.
[28] L. Bally-Cuif, M. Wassef, Determination events in the nervous system of the vertebrate embryo, Curr. Opin. Genet. Dev. 5 (1995) 450–458.
[29] M.H. Rosner, M.A. Vigano, K. Ozato, P.M. Timmons, F. Poirier, P.W. Rigby, L.M. Staudt, A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo, Nature 345 (1990) 686–692.
[30] P.A. Wilson, A. Hemmati-Brivanlou, Induction of epidermis and inhibition of neural fate by Bmp-4, Nature 376 (1995) 331–333.
[31] L.C. Lo, J.E. Johnson, C.W. Wuenschell, T. Saito, D.J. Anderson, Mammalian *achaete-scute* homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells, Genes Dev. 5 (1991) 1524–1537.
[32] Q. Ma, C. Kintner, D.J. Anderson, Identification of neurogenin, a vertebrate neuronal determination gene, Cell 87 (1996) 43–52.
[33] K. Tomita, K. Moriyoshi, S. Nakanishi, F. Guillemot, R. Kageyama, Mammalian *achaete-scute* and *atonal* homologs regulate neuronal versus glial fate determination in the central nervous system, EMBO J. 19 (2000) 5460–5472.
[34] M. Nieto, C. Schuurmans, O. Britz, F. Guillemot, Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors, Neuron 29 (2001) 401–413.
[35] Y. Sun, M. Nadal-Vicens, S. Misono, M.Z. Lin, A. Zubiaga, X. Hua, G. Fan, M.E. Greenberg, Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms, Cell 104 (2001) 365–376.
[36] M.H. Farah, J.M. Olson, H.B. Sucic, R.I. Hume, S.J. Tapscott, D.L. Turner, Generation of neurons by transient expression of neural bHLH proteins in mammalian cells, Development 127 (2000) 693–702.
[37] R.G. Phillips, J.R.S. Whittle, *Wingless* expression mediates determination of peripheral nervous system elements in late stages of *Drosophila* wing disc development, Development 118 (1993) 427–438.
[38] J.P. Couso, S.A. Bishop, A. Martinez-Arias, The *wingless* signaling pathway and the patterning of the wing margin in *Drosophila*, Development 120 (1994) 621–636.