Cell Mol Neurobiol (2009) 29:55–67
DOI 10.1007/s10571-008-9295-9

ORIGINAL PAPER

DIXDC1 Promotes Retinoic Acid-Induced Neuronal Differentiation and Inhibits Gliogenesis in P19 Cells

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Received: 17 April 2008 / Accepted: 20 June 2008 / Published online: 16 July 2008

Abstract Human DIXDC1 is a member of Dishevelled-Axin (DIX) domain containing gene family which plays important roles in Wnt signaling and neural development. In this report, we first confirmed that expression of Ccd1, a mouse homologous gene of DIXDC1, was up-regulated in embryonic developing nervous system. Further studies showed that Ccd1 was expressed specifically in neurons and colocalized with early neuronal marker Tuj1. During the aggregation induced by RA and neuronal differentiation of embryonic carcinoma P19 cells, expressions of Ccd1 as well as Wnt-1 and N-cadherin were dramatically increased. Stable overexpression of DIXDC1 in P19 cells promoted the neuronal differentiation. P19 cells overexpressing DIXDC1 but not the control P19 cells could differentiate into Tuj1 positive cells with RA induction for only 2 days. Meanwhile, we also found that overexpression of DIXDC1 facilitated the expression of Wnt1 and bHLHs during aggregation and differentiation, respectively, while inhibited gliogenesis by down-regulating the expression of GFAP in P19 cells. Thus, our finding suggested that DIXDC1 might play an important role during neurogenesis, overexpression of DIXDC1 in embryonic carcinoma P19 cells promoted neuronal differentiation, and inhibited gliogenesis induced by retinoic acid.

Keywords DIXDC1 · Ccd1 · P19 Cells · Retinoic acid · Neuronal differentiation · Gliogenesis

XT Jing and HT Wu contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s10571-008-9295-9) contains supplementary material, which is available to authorized users.

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Introduction

DIXDC1 is a protein containing a coiled-coil domain and a Dishevelled-Axin (DIX) domain. It has been identified as a positive regulator in Wnt signaling pathway. Overexpression of its zebrafish homolog Ccd1 in zebrafish embryos leads to a reduction of the size of the eyes and forebrain as a result of Wnt pathway activation, whereas a dominant-negative form of zebrafish Ccd1 (DN-Ccd1) overexpression causes the opposite phenotype (Shiomi et al. 2003;

Shiomi et al. 2005). The Wnt signaling pathway is one of the major developmental signaling pathways regulating important processes such as cell proliferation, polarity, and specification of cell fate (Wodarz and Nusse 1998). Many evidences showed that the Wnt signaling may function as a neural determination factor in the morphogenesis of the neural tube and/or the early stages of CNS development. As a positive regulator of Wnt signaling pathway, DIXDC1 was supposed to have a role in the neuronal development. In addition, an isoform of DIXDC1 is also shown to be a cytoskeletal-associated protein that may be involved in modulating dynamics of actin filaments (Wang et al. 2006). These observations indicate that DIXDC1 may play a role in cell proliferation and differentiation.

P19 cell is a mouse embryonic carcinoma (EC) cell line, which could be induced by retinoic acid (RA) to differentiate into neuroectodermal cell lineages, such as neurons and glial cells (McBurney et al. 1982; Jones-Villeneuve et al. 1982). The role of RA in the neuronal differentiation in the nervous system has been studied extensively in vitro models including embryonal carcinoma P19 cells, which has been used as a model system for studying early embryonic development and differentiation. The treatment of aggregated P19 cells with higher concentrations (1 μM) of RA results in their differentiation into neurons and glias (Jones-Villeneuve et al. 1982) by activating the transcription of many genes, including ones that encode transcription factors, cell signaling molecules, structural proteins, enzymes, and cell-surface receptors (Maden 2001).

In this paper, we first produced homemade rabbit polyclonal antibody to Ccd1 and confirmed that the high expression of mouse Ccd1 during the embryo development and the localization of Ccd1 in early neurons which were positive for neuronal marker Tuj1. Thus, Ccd1 was estimated to play a role in the developing nervous system at the initial stage of the embryo (E9.5–E15.5). Then we investigated the expression of endogenous Ccd1 and the role of overexpression of exogenous DIXDC1 during neuronal differentiation of P19 cells. The expression of endogenous Ccd1 was increased during the neuronal differentiation of P19 induced by RA for 4 days. By using of the stable cell line overexpressing human full-length DIXDC1, we demonstrated that overexpression of human DIXDC1 could not directly initiate neuronal differentiation of P19 cells without RA induction, but constitutive expression of human DIXDC1 in P19 cells could facilitate the expression of Wnt1 during aggregation and promote the neuronal differentiation while inhibiting the glial differentiation through regulating the expression of basic helix-loop-helix (bHLH) neuronal regulatory genes and glial fibrillary acidic protein.

Experimental Procedure

Materials

The restriction enzymes and other modifying enzymes for DNA manipulation were purchased from TaKaRa (Kyoto, Japan). The anti-mouse β-III-Tubulin (Tuj1) and anti- rabbit polyclonal antibody of microtubule-associated protein 2 (MAP2) were purchased from Upstate (San Francisco, CA, USA); chicken anti-GFP antibody was purchased from Abcam (Abcam, UK); mouse monoclonal antibodies including anti-NeuN and anti-glial fibrillary acidic protein (GFAP) were purchased from Chemicon (Chemicon Temecula, CA, USA). Anti-mouse and anti-rabbit IgG-alkaline phosphatase conjugates were from Promega (Madison, WI, USA). All other reagents used were from Invitrogen and Sigma.

Generation of Anti-Ccd1 Polyclonal Antibody

Female New Zealand white rabbits were immunized separately with 1 mg of GST-fused DIXDC1 fragment (amino acids 1–220) in phosphate-buffered saline with complete Freund's adjuvant. Three weeks later, the rabbits were boosted with the same proteins with incomplete Freund's adjuvant every 2 weeks for a total of three times. The polyclonal antibodies were affinity purified and characterized as shown in results.

Whole-Mount In situ Hybridization

Embryos were prepared for whole-mount in situ hybridization as previously described (Conlon and Rossant 1992). Messenger RNA (mRNA) transcripts were detected using Digoxigenin-labeled 380-bp riboprobes which was subcloned into pBluescript-SK (Stratagene) and synthesized using T3 or T7 polymerases. The probe used revealed reproducible hybridization patterns when in antisense orientation

and no signal in the sense orientation. Photographs of whole-mount stained embryos were taken with a Leica MZ8 stereomicroscope.

Preparation of Expression Plasmids

The human DIXDC1 full-length cDNA plasmid pBlueScriptII-KIAA1735 was generously provided by Kazusa DNA Research Institute, Japan. The coding sequence of DIXDC1 (Genebank: AB051522) was subcloned into the mammalian expression vector pcDNA4/*myc*-His to construct the recombinant vector pcDNA4-DIXDC1.

Cell Culture and Transfection

P19 EC cells were obtained from the American Tissue Culture Center (Rockville, USA) and cultured in α-modified Eagle's medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum under a humidified atmosphere with 5% (v/v) CO_2 in air at 37°C. To induce neuronal 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 μM all-trans retinoic acid (RA, Sigma) in 10% fetal bovine serum (FBS)/α-MEM. After 4 days of aggregation, cells were dissociated into single cells by 0.25 trypsin and were replated in a poly-L-lysine coated tissue culture dish at a density of 2×10^5 cells/ml in neuronal culture medium (DMEM/F12 supplemented with 5 μg/ml human transferrin, 20 nM progesterone, 60 μM putresine, and 30 nM sodium selenite). The cells were then allowed to adhere and cultured for 2–3 weeks with replacement of the medium every 48 h. For stable transfection, P19 cells were transfected with pcDNA4 and pcDNA4-DIXDC1 which encoded full-length human DIXDC1 gene by using Lipofectamine2000 (Invitrogen, USA). The transfected P19 cells were selected in the presence of the zeocin (600 μg/ml) for 2 weeks, and the medium was changed every 3 days. The zeocin-resistant clones were harvested and analyzed.

RNA Isolation and Semiquantitative RT-PCR

Total RNA was isolated from cell cultures using TRIzol reagent (Invitrogen). For RT-PCR, preamplification system was used to reverse transcribe total RNA (2 μg) into complementary DNA according to manufacturer's instructions (TaKaRa). An aliquot (2 μl) of the reverse transcription reaction was subjected to PCR for different target gene, respectively. PCR reactions were performed according to the user manual. The sequences of the primers used are listed in Table 1. β-actin was included as the internal control for each cDNA sample. The PCR products were electrophoresed on 2% agarose gel containing 0.5 μg/ml of ethidium bromide.

Immunofluorescence Analysis of Colocalization in Neurons

The primary cultured neurons derived from new born mouse hippocampus were fixed with 4%

Table 1 List of mouse-specific PCR primers and their sequences used in the study

Gene	Forward	Reverse	Annealing temperature (°C)	Cycles
N-cadherin	5′-atgcggatgatccaaatgccc-3′	5′-catggcagtaaactctggagg-3′	57	26
Wnt-1	5′-acgttgctactggcactgac-3′	5′-ccatttgcactctcgcacag-3′	57	30
DIXDC1	5′-atccatcaagcaccatcaac-3′	5′-cccaaactttcccagtcgt-3′	58	28
Ccd1	5′-gaacagatgagccagactct-3′	5′-cctgattctcctccacactc-3′	60	30
NF-160	5′-aggcagccaaagtgaaca-3′	5′-actcggaccaaagccaat-3′	58	28
Ngn-1	5′-cctttggagacctgcatctc-3′	5′-gatgtagttgtaggcgaagc-3′	52	28
Mash-1	5′-caagttggtcaacctgggtt-3′	5′-gctcttgttcctctgggcta-3′	56	30
BMP4	5′-tgccgcagcttctctgagcc-3′	5′-gctctgccgaggagatcacc-3′	64	26
Oct-4	5′-cagaagaggatcaccttggg-3′	5′-gtgagtgatctgctgtaggg-3′	60	28
GFAP	5′-tttctccttgtctcgaatga-3′	5′-ggtttcatcttggagcttct-3′	55	30
Zeocin	5′-caagttgaccagtgccgt-3′	5′-acacgacctccgaccact-3′	57	28
β-actin	5′-tcgtcgacaacggctccggcatgt-3′	5′-ccagccaggtccagacgcaggat-3′	56	20

paraformaldehyde followed by the treatment with 0.1% TritonX-100. For detection of Ccd1 and Tuj1 colocalization, fixed cells were washed in 0.1 M phosphate-buffered saline (PBS) and incubated with anti-Tuj1 and self-generated anti-Ccd1 polyclonal antibodies using an appropriate blocking serum (5% normal goat serum) at 4°C overnight. After washing in PBS for three times, secondary antibodies fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and TRITC-conjugated goat anti-mouse IgG were used to visualize the antigens.

Immunohistochemistry and Immunocytochemistry Analysis

E11.5 and E13.5 frozen mouse embryos were sectioned in 10 μm and fixed to glass slides in 4% formaldehyde solution for 10 min. To block non-specific binding, the sections were incubated with 5% horse or goat serum in phosphate-buffered saline (PBS; pH 7.4) for 1 h at room temperature. The sections were incubated with rabbit anti-Ccd1 antibodies (1:500) and mouse anti-Tuj1 (1:1000), respectively in PBS containing 5% horse serum and 0.1% Triton X-100 at 4°C overnight. After washing three times in PBS, the sections were incubated with anti-rabbit and anti-mouse secondary antibodies conjugated with HRP for 2 h, and the immunoreactivity was visualized by using diaminobenzidine (DAB) as a chromogen.

After RA induction, P19 cells plating on glass coverslips in multiwell dishes were induced to differentiate, and then fixed with either 4% paraformaldehyde followed by the treatment with 0.1% TritonX-100. The differentiation of P19 cells were examined with immunostaining of Tuj1 (1:1000), microtubule-associated protein2 (MAP2) (1:500), NeuN (1:1000), and glial fibrillary acidic protein (GFAP) (1:2000) as described above. After washing three times in PBS and incubated with HRP-labeled respective secondary antibodies, the diaminobenzidine (DAB) was also used to visualize the immunoreactive antigens.

Western Blot Analysis

Whole cell or murine embryos tissue lysates were prepared by resuspending them in lysis buffer (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton-X100, 10% glycerol) supplemented with protease inhibitors on ice for 30 min and spinning down cell debris at 10,000*g* for 10 min at 4°C. The concentration of protein was measured using Bradford protein assay reagent (Bio-Rad) in a Beckman Coulter DU800 spectrometer. Eighty micrograms of total protein per sample was loaded into 12% Tris/Glycine gels, electrophoresed, and then electroblotted onto PVDF membranes. Blots were blocked in 5% non-fat dry milk dissolved in Tris-Buffered Saline (TBS pH 7.4) containing 0.1% Tween-20 (TBST) for 1 h, probed overnight with primary antibodies including anti-GFP (1:1000), anti-Ccd1 (1:500), and anti-GFAP (1:2000), washed in TBST, followed by horseradish peroxidase-coupled secondary antibodies and developed by a chemiluminescence-based detection system (ECL, Amersham Pharmacia Biotech.), and photographed. Anti-β-actin antibody was used to verify equal loading.

Results

Expression of Ccd1 in Mouse Nervous System is Developmental Related

The expression of Ccd1 transcripts in embryonic mouse cerebrums from E9.5 to E15.5 were detected by RT-PCR. It was reported that 14 putative mRNAs coding for multiple murine Ccd1 proteins mainly divided into Ccd1A, Ccd1B, and Ccd1C three isoforms (Shiomi et al. 2005). Of the total isoforms, murine Ccd1B is the homologous gene to human DIXDC1. According to the BLAST analysis, the primers' set we used for RT-PCR analysis were able to amplify majority isoforms of the transcripts of Ccd1 except for Ccd1C isoform. It was shown that the transcripts of Ccd1 were up-regulated in mouse cerebrum from E10.5 embryonic day (Fig. 1A). Whole-mount in situ hybridization results in E11.5 murine embryo showed that specific high level expression of Ccd1 was observed in central nervous system, such as mesencephalon, telencephalon, rhombencephalon, diencephalon, optic primordium, and spinal cord areas (Fig. 1A).

To investigate the expression of Ccd1 in translational level, a rabbit polyclonal antibody against polypeptide epitope a.a. 1–220 in DIXDC1 was generated and affinity-purified. The antibody was

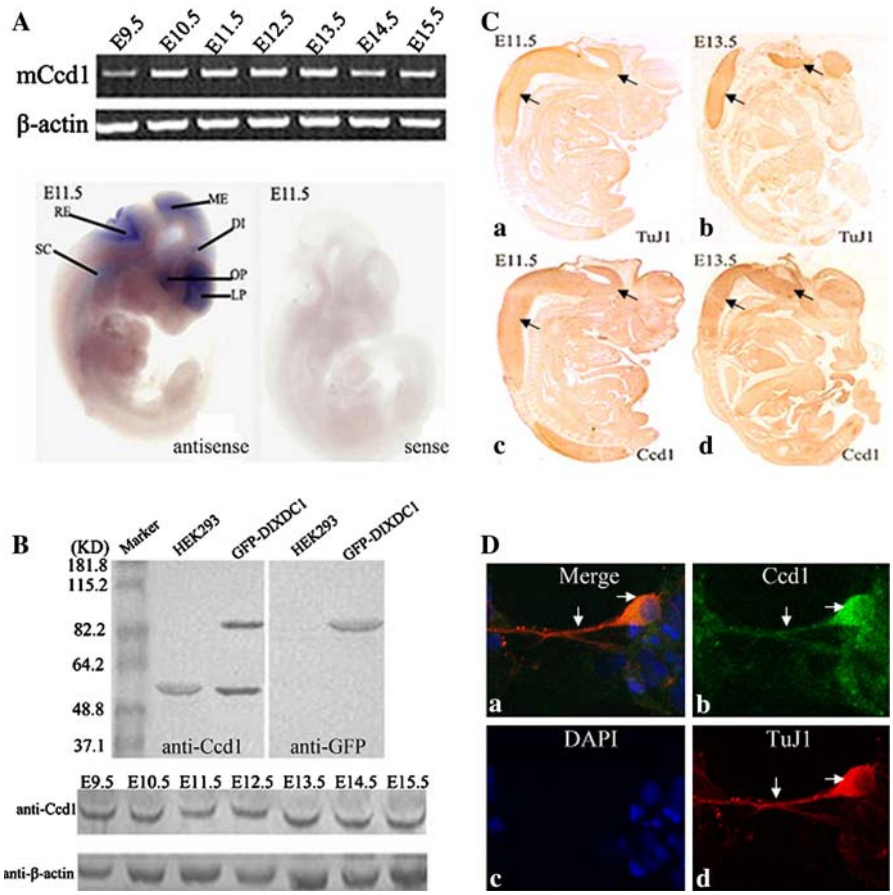

Fig. 1 Distributions of Ccd1 during embryonic development. (**A**) The expression of Ccd1 in embryonic mouse from E9.5 to E15.5 was detected by RT-PCR (upper panel) and whole-mount in situ hybridization. Only the representative result of E11.5 embryo whole-mount in situ hybridization was shown currently. It was shown that the positive signal could be detected specifically in mesencephalon (ME), telencephalon (LP), rhombencephalon (RE), diencephalons (DI), optic primordium (OP) and spinal cord (SC) areas with Ccd1-specific antisense probes (lower panel). (**B**) The specificity and sensitivity of affinity-purified anti-Ccd1 polyclonal antibodies were detected in pEGFP-DIXDC1 transfected and wild-type HEK293 cells. Two bands including 54 kD endogenous DIXDC1 and 84 kD ectopic expressed GFP-DIXDC1-fused protein could be detected by anti-Ccd1 antibodies (upper panel). The expression of Ccd1 in embryonic mouse from E9.5 to E15.5 was persistent during the embryo cerebrum development which was detected by immunoblotting with anti-Ccd1 antibodies (lower panel). (**C**) Comparison with distributions of Ccd1 and Tuj1 in mouse embryos. Immunostaining for Tuj1 (a, b) and Ccd1 (c, d) were performed in adjacent sections of E11.5 (a, c) and E13.5 (b, d) embryo, respectively. Arrowheads indicated brain and spinal cord areas where Ccd1 were coincidently coexpressed with neuron marker Tuj1. (**D**) Immunofluorescence analysis showed the colocalization of Ccd1 and Tuj1 in primarily cultured hippocampal neurons (a). Arrowheads indicated the colocalization was distributed both in somatic and axonal sites of the neuron (b, d). Nucleus of the primarily cultured hippocampal neurons were stained with DAPI blue (c)

first tested by Western blot analysis of HEK293 cells with ectopic expression of GFP-tagged DIXDC1. As shown in Fig. 1B, anti-Ccd1 antibody could recognize both the endogenous DIXDC1 and ectopic expressed GFP-DIXDC1-fused protein. Besides, the antibody was also further analyzed by immunoblotting with mouse multiple tissue extracts. It was shown that almost only a major band about 53 kDa was strongly detected which appears to correspond to DIXDC1 homologous gene Ccd1B isoform (Supplementary Fig. 1). This data showed that the rabbit polyclonal antibody we had generated possessed high specificity and sensitivity. Next, by using of this polyclonal antibody, we examined the expression patterns of Ccd1 during the mouse embryo cerebrum development. As shown in Fig. 1B, the mouse Ccd1

proteins were persistently expressed during the embryo cerebrum development. In addition, we compared with expression of Ccd1 and Tuj1 both in E11.5 and E13.5 embryo sections with anti-Ccd1 and anti-Tuj1 antibodies, respectively. Data showed that Ccd1 protein was abundantly expressed in brain and spinal cord areas and coincidently coexpressed with the early neuron marker Tuj1 (Fig. 1C).

In addition, we further investigated the distribution of Ccd1 in primary cultured cells derived from new born mouse hippocampus area with double-staining immunofluorescence analysis. Consistent with the result of embryo sections in vivo, Ccd1 was also coexpressed with Tuj1 in primary cultured neurons in vitro (Fig. 1D).

Expression of Ccd1 and Neural Regulatory Genes during RA-Induced Neuronal Differentiation of P19 Cells

Murine P19 embryonal carcinoma cells could be induced to the neuroectodermal differentiation by culturing free floating embryo bodies (EBs) in defined medium containing 1 μM RA for 4 days. After 4 days induction, EBs were digested and plated into culture dishes with neuronal culture mediums. Extensive morphological differentiation occurred 2 days later (Fig. 2A). After 5 days, cells with neuron-like morphology appeared, reaching high expression levels of neuron-specific proteins after 7–9 days as detected by staining with antibodies against β-III tubulin (Tuj1), MAP2, NeuN, and GFAP, respectively (Fig. 2B).

With the establishment of neuronal differentiation in P19, we examined the expression patterns of Ccd1 as well as some regulatory genes during RA-induced neuronal differentiation of P19 cells by RT-PCR and Western blot analysis. In accordance with the expression patterns of Wnt1 and N-cadherin, both of which are required for neuronal differentiation, Ccd1 mRNA was barely detected in undifferentiated P19 cells, whereas its expression was dramatically up-regulated after aggregation with RA induction (Fig. 3A). To further investigate the expression of Ccd1 as well as neuronal differentiation regulatory genes and explore the molecular mechanisms during neuronal differentiation in P19 cells, we examined the expression patterns of those developmental regulatory genes, such as N-cadherin, NF-160, Ngn-1, and GFAP. As shown in Fig. 3B, expression of Ccd1 was gradually increased during neuronal differentiation of P19, showing similar expression pattern to N-cadherin and NF-160. The expression of Ngn1 was kept at a relatively high level until day 8 after replating. It was also shown that the expression of GFAP appeared only at day 8 after replating, and which was up-regulated continuously until day 14 (Fig. 3B). With the use of anti-Ccd1 antibody, we examined the expression pattern of Ccd1 protein during the neuronal differentiation of P19 cells. It was shown that Ccd1 protein was persistently expressed from aggregation period to differentiation period, with a moderately high expression during the differentiation period (Fig. 3C).

Overexpression of DIXDC1 Promotes the Neuronal Differentiation of P19 Cells

To investigate the role of Ccd1 during the neuronal differentiation of P19 cells, P19 cells stably transfected with pcDNA4-DIXDC1 or pcDNA4 plasmids were cultured in medium containing zeocin (600 μg/ml) to select for the positive clones. Two weeks later, four clones were obtained. Total RNA extracted from the four selected clone cells were used to examine the ectopic expression of DIXDC1 with RT-PCR. It was demonstrated that all the selected clones showed the expression of exogenous DIXDC1 transcripts (Fig. 4A). Then three of the four clone cells were induced by RA and subjected for the following differentiation. It was shown that these three clones also possessed relatively higher overexpression of Ccd1 compared with control cells in protein level (Fig. 4B). All of these cells showed the enhanced aggregation induced by RA (1 μM). Pictures of clone 3 (shown as DIXDC1-P19) represent the common phenotype. As shown in Fig. 4, DIXDC1-P19 cells constitutively expressing ectopic DIXDC1 in P19 cells were able to aggregate into larger embryo bodies induced by RA only for 2 days. After being digested and replated into neuronal culture mediums as the single cell suspension, these cells could differentiate into neuronal-like cells, showing bipolar morphology with many processes at day 2, sending out long neurite-like processes by day 4, and forming neuronal networks by day 8 (Fig. 4C a1–a4). Besides, other two DIXDC1 overexpression clones also could differentiate in the similar manner (data not shown). In contrast, pcDNA4-P19 control cells were only

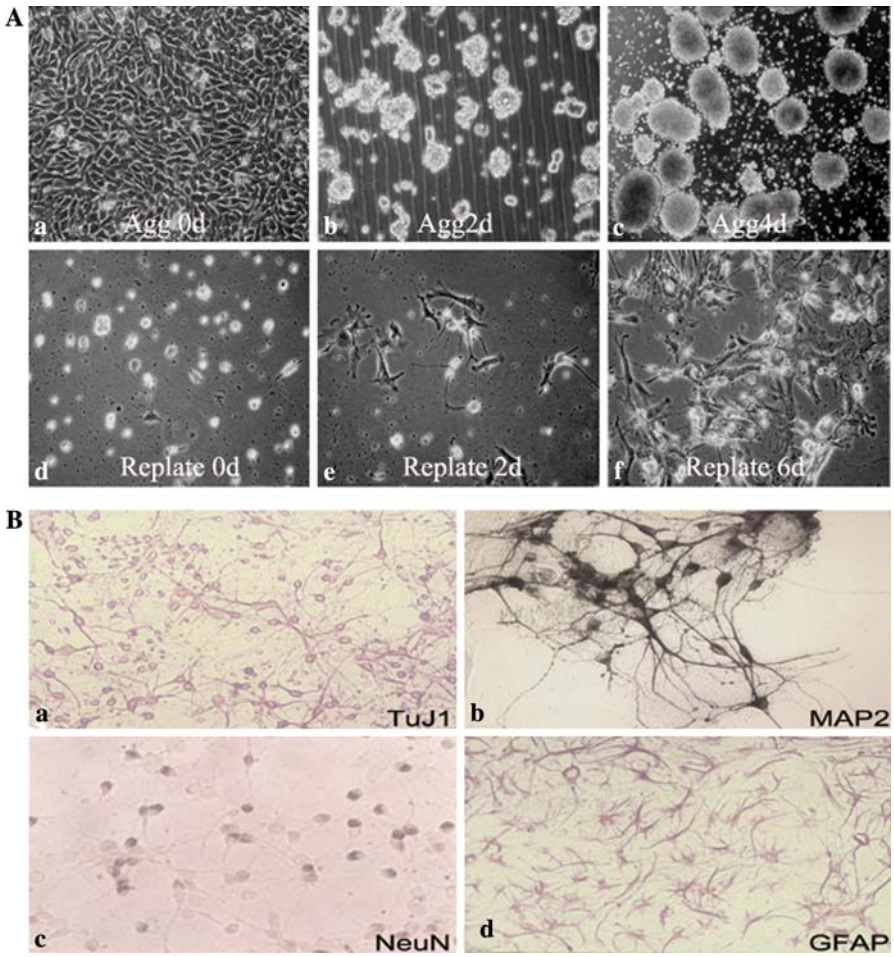

Fig. 2 Morphology and immunocytochemistry identification of P19 cells during their neural differentiation. (**A**) P19 cells were aggregated for 0 day (a), 2 days (b) and 4 days (c) with the induction of RA, then replated as the single cell suspension and cultured in neuronal culture mediums for 0 day (d), 2 days (e) and 6 days (f), respectively. Note that P19 cells formed long neurite-like processes after aggregation and RA induction. (**B**) Immunocytochemical characterization of neuron-like cells differentiated from P19 cells. The differentiated neuronal cells were stained with anti-Tuj1 (a), anti-MAP2 (b), anti-NeuN (c), and anti-GFAP (d) antibodies, respectively. Note that the differentiated neuronal cells formed long neurite-like processes and were strongly positive for neuron specific proteins: Tuj1, MAP2 and NeuN. Meanwhile, many GFAP positive glia-like cells also appeared in the population of differentiated neuronal cells after replating for 7–9 days

aggregated into smaller embryo bodies induced by RA for 2 days, and showed no differentiation phenotype even after being replated in neuronal culture mediums for 8 days (Fig. 4C b1–b4).

Moreover, by using of immunocytochemistry method, we found that the neuron-like cells only derived from DIXDC1-P19 cells with RA induction for 2 days were positive for Tuj1 staining at day 8 (Fig. 4C a5). In contrast, we almost could hardly find any Tuj1 positive neuron-like cells in the culture of pcDNA4-P19 cells even after being replated for more than 8 days (Fig. 4C b5). However, when pcDNA4-P19 cells were induced with RA for 4 days and replated in neuronal culture mediums, normal embryo bodies and neural differentiation phenotype appeared and Tuj1 positive neuron-like cells could also be found at day 8 just like wild-type P19 cells (Fig. 4C c1–5).

DIXDC1 Enhances the Expression of Neuronal Regulatory Genes and Inhibits the Expression of GFAP in P19 Cells

The previous data suggested that overexpression of the DIXDC1 gene triggered neuronal but not glial

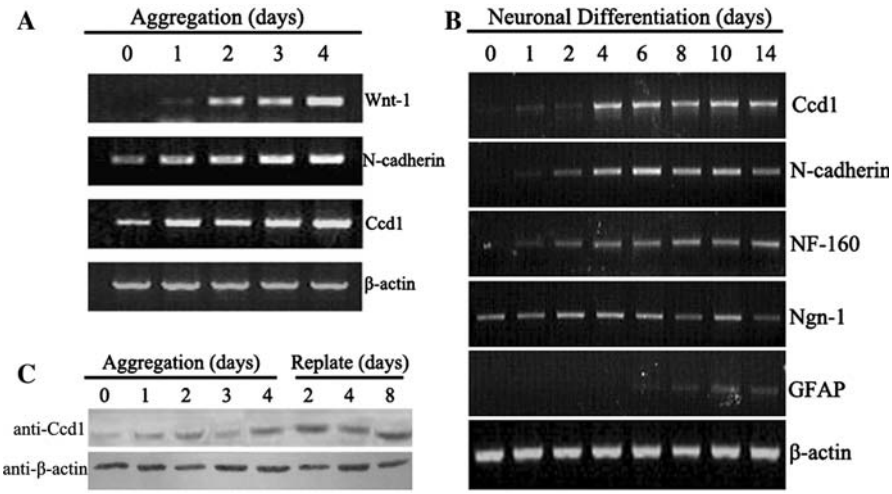

Fig. 3 Detection of the expression of Ccd1 and regulatory genes in P19 cells during RA-induced neuronal differentiation. (**A**) RT-PCR analysis of Wnt-1, N-cadherin and Ccd1 mRNA expression in P19 cells during the aggregation induced by RA. For RT-PCR analysis, samples were harvested from the cells aggregated from day 0 to day 4, respectively. (**B**) RT-PCR analysis of Ccd1, N-cadherin, NF-160, Ngn-1 and GFAP mRNA expression in differentiating P19 cells. Samples were harvested from the cells replated in neuronal culture medium for 0, 1, 2, 4, 6, 8, 10 and 14 days, respectively. (**C**) Western blot analysis of Ccd1 protein expression in differentiating P19 cells. For immunoblotting analysis, samples were prepared from cell lysates of undifferentiated cells, cells aggregated from day 1 to day 4, and cells replated in neuronal culture mediums for 2, 4 and 8 days, respectively. All experiments were repeated three times, and similar results were obtained

differentiation of P19 cells. To further explore the molecular mechanisms underlying DIXDC1-induced neuronal differentiation, RT-PCR was used to examine the expression patterns of those developmental regulatory genes, such as Oct-4 (Rosner et al. 1990), BMP-4 (Wilson and Hemmati-Brivanlou 1995), Mash-1 (Lo et al. 1991), and Ngn-1 (Ma et al. 1996; Farah et al. 2000) in DIXDC1-P19 and pcDNA4-P19 control cells (Fig. 5). Among these genes, expression pattern of Oct-4, in general, was similar between these cells. The expression of BMP-4 was moderately up-regulated in differentiated DIXDC1-P19 cells (Fig. 5A). Mash-1 and Ngn-1 were not expressed in both DIXDC1-P19 cells and pcDNA4-P19 control cells in their undifferentiated state. In pcDNA4-P19 cells, the expression of Mash-1 and Ngn-1 started at day 1 and day 2, respectively, after RA induction and maintained at a rather low level during the induction and differentiation stage. In contrast to pcDNA4-P19 cells, in DIXDC1-P19 cells, the expression of Mash-1 and Ngn-1 were only detected during the differentiation process and maintained at a relatively high level (Fig. 5A). Taken together, these results show that in DIXDC1-P19 cells the bHLH genes, such as Mash-1 and Ngn-1, start to express only in the neural differentiation stage, which suggest that these neural bHLH genes need to be involved in the neural differentiation of DIXDC1-P19 cells.

In addition, we also investigated the effect of DIXDC1 on the expression of glial marker GFAP. In accordance with the phenotype that DIXDC1-P19 cells could not differentiate into glia cells after being replated for 8 days, the expression of GFAP both in mRNA and protein levels could not be detected at all in DIXDC1-P19 cells as late as day 8 after being replated (Fig. 5B, C).

Discussion

Although many literatures have been published about the study of Ccd1 gene in the past few years, the exact role of Ccd1 during neuronal development is still unclear. In this study, we have shown that Ccd1 expression is highly related to embryonic development. RT-PCR analysis gave the definite result that pan-Ccd1 mRNA expression was up-regulated from E10.5 and maintained a high expression level till E13.5. The whole-mount in situ hybridization results confirmed the high expression of Ccd1 in the cerebrum of E11.5 embryo. Several positive signals

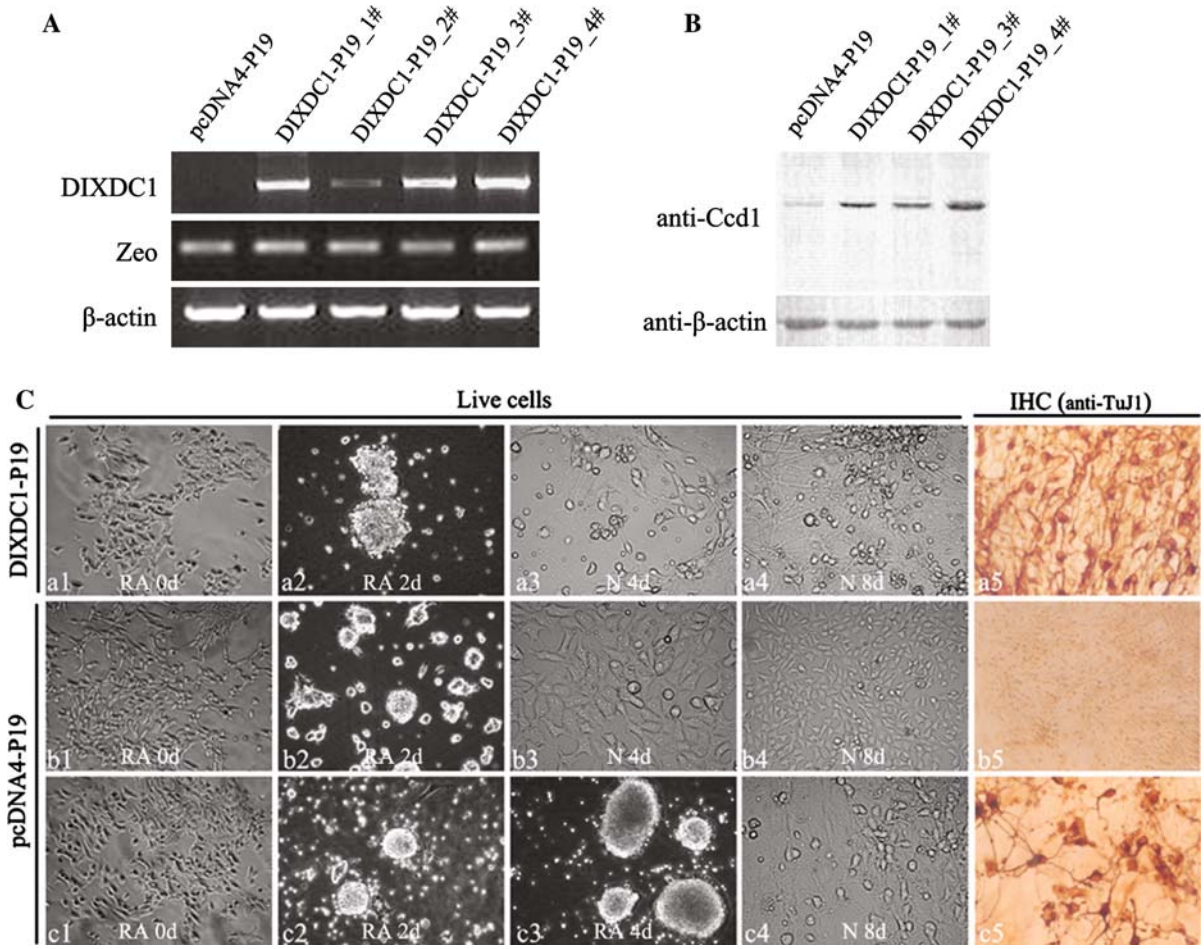

Fig. 4 Overexpression of DIXDC1 promoted the neuronal differentiation of P19 cells. (**A**) Four selected clones of DIXDC1-P19 cells and pcDNA4-P19 control cells were amplified by RT-PCR to confirm the overexpression of exogenous DIXDC1. Zeocin resistance gene contained in pcDNA4/myc-His vector was also amplified. (**B**) Western blot analysis of DIXDC1 overexpression in three of the four selected clones of DIXDC1-P19 cells and pcDNA4-P19 control cells. (**C**) DIXDC1-P19 (a1) and pcDNA4-P19 (b1) cells were induced with RA for 2 days (a2, b2) and then replated in neuronal culture mediums to induce neuronal differentiation for 4 days (a3, b3) and 8 days (a4, b4), respectively. In contrast with the formation of plenty of Tuj1 positive neuron-like cells in DIXDC1-P19 cells (a5), pcDNA4-P19 cells could hardly differentiate into Tuj1 positive neuron-like cells (b5). However, immunocytochemistry data demonstrated that Tuj1 positive neuron-like cells appeared again (c5) when pcDNA4-P19 cells were induced with RA for 4 days (c1–c3) and replated in neuronal culture mediums to induce neuronal differentiation for 8 days (c4)

were observed in mesencephalon, telencephlon, rhombencephalon, diencephalon, optic primordium, and spinal cord areas. These results were consistent with that reported by Shiomi (Shiomi et al. 2003). Immunohistochemistry analysis with the E11.5 and E13.5 embryo sections showed that Ccd1 protein shared the similar expression pattern with the early neuron marker protein Tuj1. Further investigation on primary neuronal cells exhibited that Ccd1 was coexpressed with Tuj1 in primary neurons. It should be emphasized that our homemade rabbit polyclonal antibody could recognize both Ccd1B and Ccd1C isoforms, especially specific for about 53 kDa Ccd1B. These results indicated that Ccd1 might play an important role in the development of mouse central nervous system.

The P19 cell line was established previously as a model for in vitro neuronal differentiation. P19 cells constitute those cells of the inner cell mass of the blastocyst during the initial period of development,

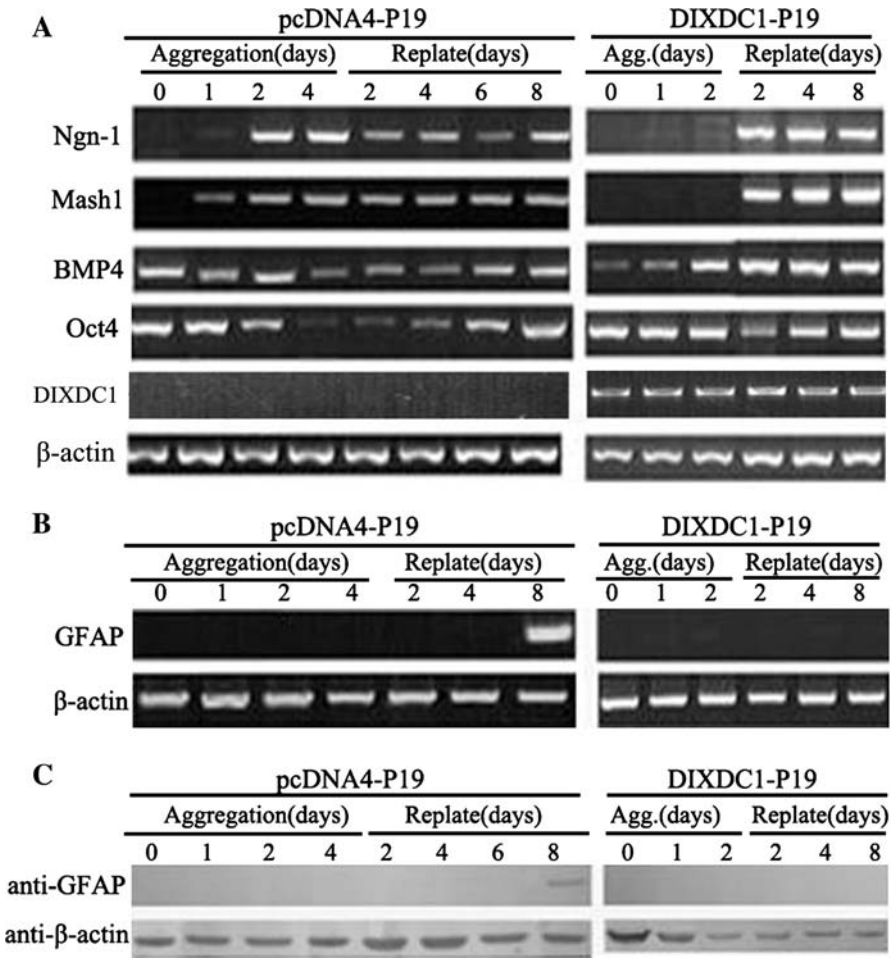

Fig. 5 Expression profiles of regulatory genes and glial cell marker GFAP during pcDNA4-P19 and DIXDC1-P19 cells neural differentiation. (**A**) RT-PCR analysis of regulatory genes such as Mash-1, BMP-4, Oct-4, and Ngn-1 mRNA expression in differentiating pcDNA4-P19 and DIXDC1-P19 cells. Human DIXDC1 was also amplified to confirm the overexpression of exogenous DIXDC1 transcripts in DIXDC1-P19 cells. Samples were harvested at the indicated times after RA-induced aggregation and neuronal differentiation. (**B**) RT-PCR analysis of glial cell marker GFAP mRNA expression in differentiating pcDNA4-P19 and DIXDC1-P19 cells. Samples were harvested at the indicated times after RA-induced aggregation and neuronal differentiation. (**C**) Western blot analysis of GFAP protein expression in differentiating pcDNA4-P19 and DIXDC1-P19 cells at the indicated times after RA-induced aggregation and neuronal differentiation. All experiments were repeated three times, and similar results were obtained

and the differentiation of P19 cells is believed to closely mimic critical events during early embryogenesis. RA treatment induces P19 cells to differentiate into ectoderm-derived neural cells including neurons and astrocytes (McBurney et al. 1982; Jones-Villeneuve et al. 1982). According to the treatment of cells, the neuronal 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 4 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 this stage, some genes or pathways need to be up-regulated or activated for differentiation to occur. Although several candidates have emerged such as Wnt1, N-cadherin, and Sox6 (Bain et al. 1996; Tang et al. 2002; Hamada-Kanazawa et al. 2004), much more research need to be focused on this area. Here, we fortunately found that the expression

of Ccd1 was up-regulated during the aggregation of P19 cells induced by RA both at transcriptional and translational levels. Thus, these results suggested that Ccd1 might participate in the neuronal differentiation of P19 cells induced by two elements including RA stimulation and cellular aggregation. Besides, we also found that overexpression of DIXDC1 in P19 cells could facilitate and promote the expression level of Wnt1a during RA-induced aggregation (Supplementary Fig. 2). 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 (Jones-Villeneuve et al. 1982; MacPherson and McBurney 1995). It was shown that the mRNA transcription and protein translation of Ccd1 were both induced to express from the second day of RA induction and maintained a relatively high expression level during the differentiation. Together, our results demonstrated that Ccd1 might participate in the aggregation and Wnt signaling transduction pathway of RA-induced neuronal differentiation of P19 cells.

After confirming the involvement of Ccd1 during the neuronal differentiation of P19, we established the DIXDC1-P19 cells stably overexpressing DIXDC1 to confirm the effects of DIXDC1 on the neuronal differentiation of P19 cells. Normally, wild-type P19 cells could be induced to differentiate into neuronal cells in the present of RA for 4 days. But DIXDC1-P19 cells overexpressing exogenous DIXDC1 could form the EBs with the induction of RA for only 2 days. When being replated into the neuron culture dishes, the induced DIXDC1-P19 cells could differentiate into Tuj1 positive neuronal cells and showed the neuronal network at day 8. In parallel, the pcDNA4-P19 cells induced by RA for only 2 days could not differentiate to neuronal cells even cultured in neuronal culture mediums for 8 days. As the neurogenesis in vivo, neurons appear earlier than glial cells during RA-induced P19 cell neuronal differentiation (Bally-Cuif and Wassef 1995). Interestingly, our results show that DIXDC1-P19 cells differentiate restrictively into neurons, but not glial cells. In contrast to the plenty of neuronal network, we could not find glial cells in the DIXDC1-P19 cultures even after being replated in neuronal mediums for 8 days. At the same time, the expression of glial cell marker GFAP could not be detected at the later stage of differentiation of DIXDC1-P19 cells even without adding any mitotic inhibitor Cytarabine, which is used to inhibit the growth of glial cells. These results demonstrated that overexpression of DIXDC1 could promote the neuronal and inhibit the glial differentiation of P19 cells induced by RA.

It was previously reported that Wnt-1 could promote the neuronal fate and inhibit glial fate in P19 cells through activation of neural bHLH gene expression (Tang et al. 2002). Neural bHLH genes have 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 (Tomita et al. 2000). 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 (Nieto et al. 2001). As a transcriptional activator, Ngn-1 has been demonstrated to promote neurogenesis and inhibit astrocyte differentiation by sequestering the CBP-Smad transcription complex away from astrocyte differentiation genes and inhibiting the activation of STAT transcription factors (Sun et al. 2001). Thus, we further investigated the possible molecular events involved in DIXDC1 promoted differentiation of P19 cells. In parallel, the expression of two neural bHLH genes, Mash-1 and Ngn-1, were both suppressed during aggregation stage and up-regulated during the differentiation stage by ectopic expression of DIXDC1 in P19 cells. This suggested that DIXDC1 might also promote the neuronal fate and inhibit glial fate in P19 cells in the similar manner with Wnt-1 by activation of neural bHLH gene expression in differentiation stage.

It has been demonstrated that overexpression of N-cadherin, MEF2C, and Wnt-1 could substitute for the role of RA, but not the role of cellular aggregation in the initial stage of neuronal differentiation (Gao et al. 2001; Skerjanc and Wilton 2000). Besides, although we have demonstrated that overexpression of DIXDC1 promotes the neuronal differentiation and inhibits glial fate in P19 cells by up-regulating Mash-1 and Ngn-1 bHLH genes and Wnt1a, overexpression of DIXDC1 could not substitute for the role of both RA and cellular aggregation, because DIXDC1-P19

cells could not differentiate into neuronal cells without RA induction. In addition, considering the reason why bHLHs are suppressed by ectopic expression of DIXDC1 during aggregation, we speculate that it may be correlated with the up-regulated expression of Wnt1 induced by exogenous DIXDC1, because previous work had demonstrated that both of Mash-1 and Ngn-1 were suppressed during aggregation in Wnt1 overexpressing P19 cells (Tang et al. 2002). On the other hand, it was reported that overexpression of NeuroD2, Mash-1, Ngn-1, or other bHLH genes is sufficient to convert P19 cells into differentiated neurons in the absence of RA (Farah et al. 2000). As bHLHs are suppressed by DIXDC1 overexpression in P19 cells, maybe this can also explain why it is not enough for DIXDC1 overexpression in P19 cells to start the neuronal differentiation without RA induction.

As a small molecule, the role of RA in neuronal differentiation has been studied extensively in in vitro models. RA induced the differentiation of various types of neurons and glia (Andrews 1984; Sidell et al. 1983) by activating the transcription of many genes including Ngn-1 (Maden 2001). The ability of RA to induce neuronal differentiation can be harnessed to produce specific neural cells that can be used for therapeutic transplantation. Taken together, our studies provided some evidences that DIXDC1 might play an important role in the crosstalk between RA signaling pathway and bHLH genes during neuronal differentiation in P19 cells.

Acknowledgment This work was supported by grants from National Basic Research Program of China (2002BA711A01-03). We thank Kazusa DNA Research Institute for the generous gift of KIAA1735 (AB051522) human cDNA clone.

References

Andrews PA (1984) Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. Dev Biol 103:285–293. doi:10.1016/0012-1606(84)90004-6

Bain G, Ray WJ, Yao M et al (1996) Retinoic acid promotes neural and represses mesodermal gene expression in mouse embryonic stem cells in culture. Biochem Biophys Res Commun 223:691–694. doi:10.1006/bbrc.1996.0957

Bally-Cuif L, Wassef M (1995) Determination events in the nervous system of the vertebrate embryo. Curr Opin Genet Dev 5:450–458. doi:10.1016/0959-437X(95)90048-L

Conlon RA, Rossant J (1992) Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo. Development 116:357–368

Farah M, Olson J, Sucic H et al (2000) Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. Development 127:693–702

Gao X, Bian W, Yang J et al (2001) A role of N-cadherin in neuronal differentiation of embryonic carcinoma P19 cells. Biochem Biophys Res Commun 284:1098–1103. doi:10.1006/bbrc.2001.5089

Hamada-Kanazawa M, Ishikawa K, Nomoto K et al (2004) Sox6 overexpression causes cellular aggregation and the neuronal differentiation of P19 embryonic carcinoma cells in the absence of retinoic acid. FEBS Lett 560:192–198. doi:10.1016/S0014-5793(04)00086-9

Jones-Villeneuve EM, McBurney MW, Rogers KA et al (1982) Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. J Cell Biol 94:253–262. doi:10.1083/jcb.94.2.253

Lo LC, Johnson JE, Wuenschell CW et al (1991) Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. Genes Dev 5:1524–1537. doi:10.1101/gad.5.9.1524

Ma Q, Kintner C, Anderson DJ (1996) Identification of neurogenin, a vertebrate neuronal determination gene. Cell 87:43–52. doi:10.1016/S0092-8674(00)81321-5

MacPherson PA, McBurney MW (1995) P19 embryonal carcinoma cells: a source of cultured neurons amenable to genetic manipulation. Methods 7:238–252. doi:10.1006/meth.1995.1029

Maden M (2001) Role and distribution of retinoic acid during CNS development. Int Rev Cytol 209:1–77. doi:10.1016/S0074-7696(01)09010-6

McBurney MW, Jones-Villeneuve EM, Edwards MK et al (1982) Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. Nature 299:165–167. doi:10.1038/299165a0

Nieto M, Schuurmans C, Britz O et al (2001) Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. Neuron 29:401–413. doi:10.1016/S0896-6273(01)00214-8

Rosner MH, Vigano MA, Ozato K et al (1990) A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature 345:686–692. doi:10.1038/345686a0

Shiomi K, Uchida H, Keino-Masu K et al (2003) Ccd1, a novel protein with a DIX domain, is a positive regulator in the Wnt signaling during zebrafish neural patterning. Curr Biol 13:73–77. doi:10.1016/S0960-9822(02)01398-2

Shiomi K, Kanemoto M, Keino-Masu K et al (2005) Identification and differential expression of multiple isoforms of mouse coiled-coil-DIX1 (Ccd1), a positive regulator of Wnt signaling. Brain Res Mol Brain Res 135:169–180. doi:10.1016/j.molbrainres.2004.12.002

Sidell N, Altman A, Haussler MR et al (1983) Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. Exp Cell Res 148:21–30. doi:10.1016/0014-4827(83)90184-2

Skerjanc IS, Wilton S (2000) Myocyte enhancer factor 2C upregulates MASH-1 expression and induces neurogenesis

in P19 cells. FEBS Lett 472:53–56. doi:10.1016/S0014-5793(00)01438-1

Sun Y, Nadal-Vicens M, Misono S et al (2001) Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell 104:365–376. doi:10.1016/S0092-8674(01)00224-0

Tang K, Yang J, Gao X et al (2002) Wnt-1 promotes neuronal differentiation and inhibits gliogenesis in P19 cells. Biochem Biophys Res Commun 293:167–173. doi:10.1016/S0006-291X(02)00215-2

Tomita K, Moriyoshi K, Nakanishi S et al (2000) Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. EMBO J 19:5460–5472. doi:10.1093/emboj/19.20.5460

Wang X, Zheng L, Zeng Z et al (2006) DIXDC1 isoform, l-DIXDC1, is a novel filamentous actin-binding protein. Biochem Biophys Res Commun 347:22–30. doi:10.1016/j.bbrc.2006.06.050

Wilson PA, Hemmati-Brivanlou A (1995) Induction of epidermis and inhibition of neural fate by Bmp-4. Nature 376:331–333. doi:10.1038/376331a0

Wodarz A, Nusse R (1998) Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol 14:59–88. doi:10.1146/annurev.cellbio.14.1.59