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# Leucine-rich Repeat C4 Protein is Involved in Nervous Tissue Development and Neurite Outgrowth, and Induction of Glioma Cell Differentiation

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**Abstract** LRRC4, leucine-rich repeat C4 protein, has been identified in human (GenBank accession No. AF196976), mouse (GenBank accession No. DQ177325), rat (GenBank accession No. DQ119102) and bovine (GenBank accession No. DQ164537) with identical domains. In terms of their similarity, the genes encoding LRRC4 in these four mammalian species are orthogs and therefore correspond to the same gene entity. Based on previous research, and using *in situ* hybridization, we found that LRRC4 had the strongest expression in hippocampal CA1 and CA2, the granule cells of the dentate gyrus region, the mediodoral thalamic nucleus, and cerebella Purkinje cell layers. Using a P19 cell model, we also found that LRRC4 participates in the differentiation of neuron and glia cells. In addition, extracellular proteins containing both an LRR cassette and immunoglobulin domains have been shown to participate in axon guidance. Our data from neurite outgrowth assays indicated that LRRC4 promoted neurite extension of hippocampal neurons, and induced differentiation of glioblastoma U251 cells into astrocyte-like cells, confirmed by morphology observation and glial fibrillary acidic protein expression.

**Keywords** LRRC4; development; neurite outgrowth; differentiation; glioma

A growing number of leucine-rich repeat proteins (LRPs) with leucine-rich repeats (LRRs) and immunoglobulin (Ig) domains, which seem to serve similar functions, have been found to be expressed predominantly in cells of nervous system [1–3]. It is well known that the primary function of the LRR domain appears to be the provision of a versatile structural framework for the formation of protein-protein interaction [4,5]. It has been proposed that a common, phylogenetically ancient role of the Ig domain might mediate cell adhesion or recognition phenomena during nervous system development and differentiation [6,7].

Leucine-rich repeat C4 protein (LRRC4), containing LRRs and a C2-type Ig (IgC2) domain, was cloned and characterized from human chromosome 7q32 using a computer-assisted positional cloning strategy combining 5′-RACE by Wang JR *et al*. [8]. Based on the structure of LRR and IgC2, we proposed that LRRC4 might be involved in the development and differentiation of the nervous system. Based on a previous study, we detected the more specific distribution of murine (m)LRRC4 in different brain tissue using *in situ* hybridization (ISH). We also characterized the expression of mLRRC4 at different development stages and retinoic acid (RA)-induced neuronal differentiation of embryonic carcinoma P19 cells.

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## Materials and Methods

### Preparation of tissues

All the procedures involving mice were approved by

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the Animal Welfare Committee of Central South University (Changsha, China). The stage of the embryos was determined by the day of appearance of a vaginal plug and confirmed by morphological criteria. BALB/c mice were used in this study. The appearance of a vaginal plug was taken as day 0 of embryogenesis (E). Delivery of BALB/c mice takes place at E19, corresponding to the newborn stage. The mice were killed by cervical dislocation and decapitation. Mice embryos aged between E8.5 and E18.5 were dissected.

**Reverse transcription-polymerase chain reaction (RT-PCR) analysis**

Total RNA was isolated using Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, USA). After being treated with DNaseI, 1–2 μg of total RNA was reverse transcribed with oligo(dT) using a cDNA synthesis kit (Promega, Madison, USA). The subsequent PCRs were carried out using *Taq* polymerase and buffer (Promega) supplied with 0.2 mM dNTPs and 0.2 μM primers. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was amplified as the internal control to normalize the relative levels of the cDNA. The reaction products were analyzed on 1.5% agarose gels. The cDNA of neuronal differentiation of embryonic carcinoma P19 cells induced by RA was provided by Dr. Naihe JING (Institutes of Biochemistry and Cell Biology, SIBS, CAS).

**ISH**

The probe of mLRRC4 (GenBank accession No. AF290542 or DQ177325) was labeled with digoxigenin-11-dUTP by random priming and detected with monoclonal antibody to digoxigenin labeled with Biotin according to manufacturer's instructions (Boehringer Mannheim, Indianapolis, USA). The ISH detection methods have been previously described with minor modifications [9]. Briefly, 6-μm-thick sections were deparaffinized, rehydrated, digested with 20 mg/L proteinase K at 37 ºC for 20 min, and dehydrated. The slides were incubated with prehybridization solution at 37 ºC for 2 h and hybridized with mLRRC4 probe at 37 ºC overnight, then washed with graded saturated solutions of sodium citrate and treated with anti-digoxigenin horseradish peroxidase. The color was brought out by 3-amino-9-ethylcarbazole or 3,3′-diaminobenzidine, and counterstained with hematoxylin.

**Neurite outgrowth assays**

Hippocampal neurons from 18.5 d-old BALB/c mouse embryos were dissociated by trypsinization (0.125% PBS for 30 min at 37 ºC) followed by resuspension in serum-containing medium to inhibit trypsin and trituration. After three washes with PBS, cells were plated on confluent monolayers of either control U251 cells or U251 cells expressing human (h)LRRC4 [10] in 35 mm poly-L-lysine-coated tissue culture dishes at a density of 160 cells/mm$^2$. Cultures were grown in DMEM/F12 medium supplemented with 10% fetal bovine serum and 10 mM cytarabine. Forty-eight hours after plating, the cells were fixed with 4% paraformaldehyde in PBS and immunocytology was carried out using anti-microtubule associated protein-2 monoclonal antibody (Boster, Wuhan, China). Over 100 neurites were chosen for measurement. Each selected neurite could be observed emerging from a distinguishable cell body, was at least twice the length of the widest diameter of the neuronal cell body, and was identifiable over its entire length. Pictures were acquired with an Olympus BH-2 microscope (Olympus, Tokyo, Japan) equipped with a digital camera (TK-C1381; Victor Company of Japan, Tokyo, Japan). The length of each neurite was measured after tracing using an HPIAS 1000 Image Analysis System (Champion Image Engineering Company of Tongji Medical University, Wuhan, China). Neurite lengths were compared between groups using a one-way ANOVA test.

**Western blot analysis**

Western blot analysis was carried out as previously described [11]. Rabbit anti-human glial fibrillary acidic protein (GFAP) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-α-tubulin antibody (Santa Cruz Biotechnology) was used as a control.

**Indirect immunofluorescence**

Cells were fixed with cold methanol stored at –20 ºC. The cells were reacted with anti-GFAP antibody diluted 1:100 in PBS containing 0.1% bovine serum albumin overnight at 4 ºC, washed in excess PBS then reacted with 1:80 dilution of CY3-conjugated goat anti-rabbit Ig in blocking solution for 1 h at 37 ºC. Nuclear stain was revealed with 4′,6′-diamidino-2-phenylindole staining. Fluorescent images were viewed with an Olympus DP70 compact inverted fluorescence microscope with a 40× object lens and a 10× eye piece.

## Results

**Computer-assisted analysis**

hLRRC4 [8] and mLRRC4 [12] have been cloned. In

this study, we cloned rat LRRC4 [(r)LRRC4] and bovine [(b)LRRC4] by *in silico* cloning. The LRRC4 of these four mammalian species were aligned using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw/) and BOXSHADE software (http://www.ch.embnet.org/software/BOX_form.html). Homology searches with the deduced 652 amino acid residues revealed mLRRC4 shares 97% similarity with hLRRC4, 99% with rLRRC4 and 96% with bLRRC4. They all contain the conservative structure of LRR N-terminal domain-LRRs-C-terminal domain-IgC2 and share a high degree of homology in these domains (**Fig. 1**).

### More specific expression distribution of mLRRC4 mRNA in mouse post-natal brain tissue

We have reported that LRRC4 (human and mouse) displayed a brain tissue-specific expression by RT-PCR and Northern blot [12]. To examine more specific expression in brain tissue, ISH was used. We found that the mLRRC4 mRNA could be detected in neurons through-

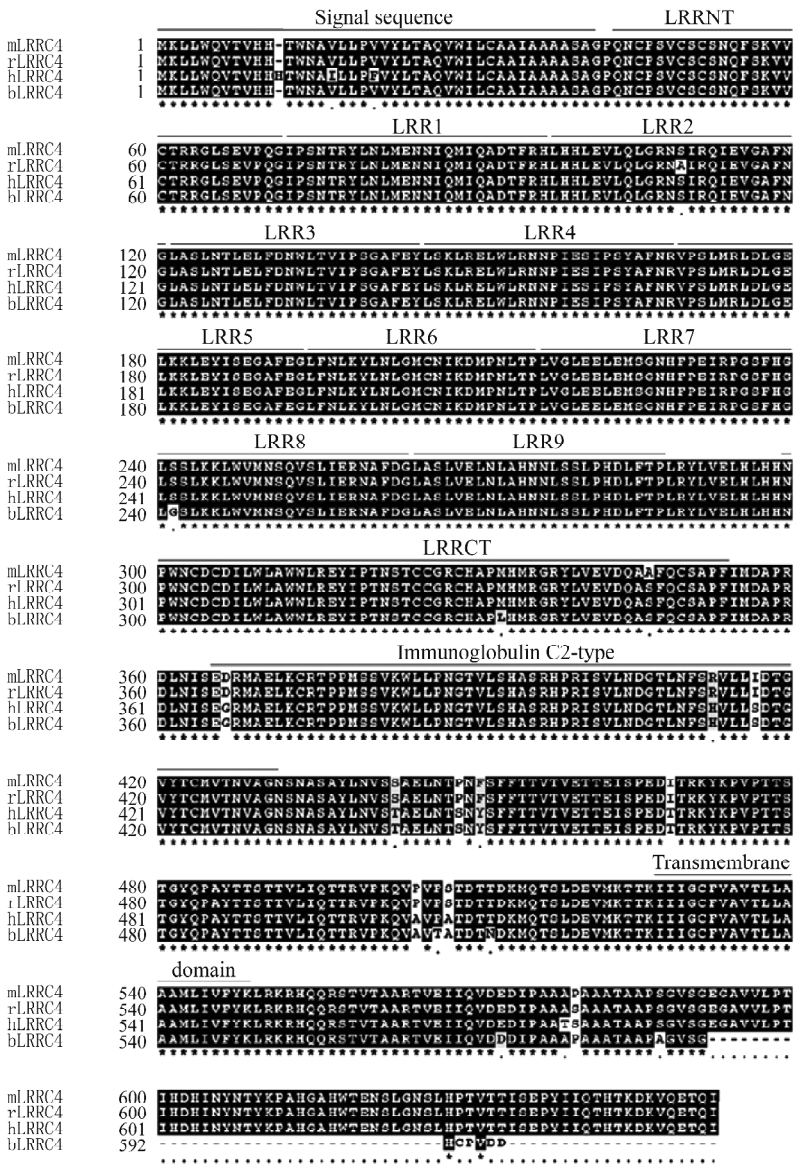


**Fig. 1 Alignment of the leucine-rich repeat protein C4 LRRC4 superfamily**

Amino acid sequence comparison of LRRC4 of mouse (mLRRC4), human (hLRRC4), rat (rLRRC4), and bovine (bLRRC4). The identical amino acids between all LRRC4s are highlighted in black, and similar amino acids are highlighted in gray. Different domains found in the members of the LRRC4 superfamily are marked above the sequences. LRRCT, leucine-rich repeat C-terminal domain; LRRNT, leucine-rich repeat N-terminal domain.

out the central nervous system, such as the cerebrum, cerebellum, spinal cord, pons and medulla oblongata. In the cerebral cortex, it was expressed in the posterior part of the prepiriform cortex, the auditory area of the temporal cortex, and the superior thalamic radiation, and the strongest expression of mLRRC4 was detected in the granule cells of hippocampal CA1 and CA2, and the dentate gyrus region [**Fig. 2(A–C)**]. It was also detected in the mediodorsal thalamic nucleus, paraventricular nucleus of thalamus, fissura hippocampi, and post-cingulated gyrus [**Fig. 2(D)**]. The transcript of mLRRC4 could also be detected throughout the cerebella cortex especially in the granular layer and the Purkinje cell layers [**Fig. 2(E)**]. In the spinal cord, mLRRC4 mRNA was mainly expressed in the gray matter [**Fig. 2(F)**]. mLRRC4 mRNA was mainly expressed in the internal granular layer [**Fig. 2(G)**] in the olfactory bulb and positive signals were also found in the epithelium of the choroids plexus [**Fig. 2(H)**]. The distribution of mLRRC4 in the liver was used as the negative control [**Fig. 2(I)**].

## mLRRC4 is involved in nervous system development and differentiation

Research, using RT-PCR, showing that LRRC4 is involved in fetal brain development has been reported previously [12]. In the present study, ISH was used to investigate the expression pattern of the mLRRC4 gene in the 12.5 d embryo mouse. In the 8 μm thick sagittal sections, the strong positive stain with mLRRC4 was found in the telencephalon [**Fig. 3(A)**, plate a], mesencephalon [**Fig. 3 (A)**, plates a,b], myelencephalon [**Fig. 3(A)**, plates a,c], choroid plexus [**Fig. 3(A)**, plates a,c] and neural tube [**Fig. 3(A)**, plates a,d]. mLRRC4 was expressed most strongly in the dorsal root ganglia [**Fig. 3(A)**, plates a,e]. However, in the other tissue, such as heart and liver [**Fig. 3(A)**, plate a], a weak signal or no signal was detected. No signal was

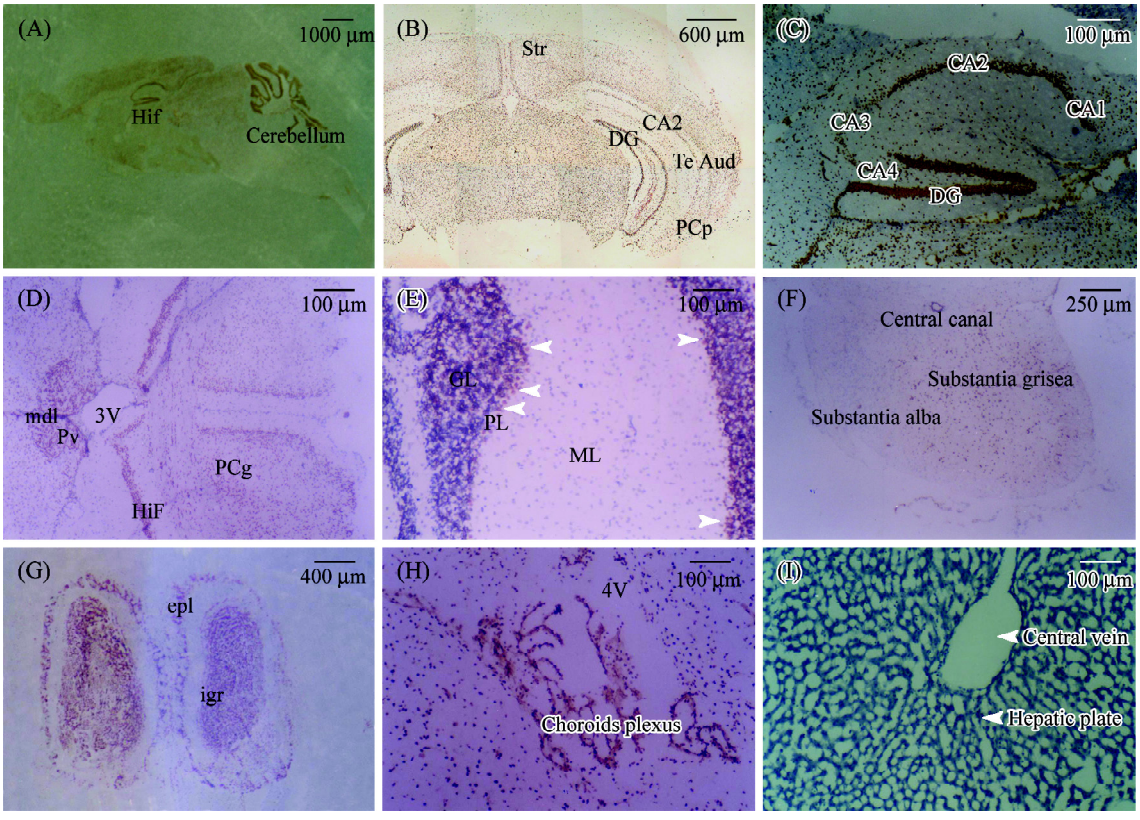


**Fig. 2** ***In situ*** **hybridization distribution of murine leucine-rich repeat C4 protein (mLRRC4) in mouse brain slices (substrate 3,3'-diaminobenzidine)**

(A–I) Brain; cerebrum; hippocampus; thalamencephalon; cerebellum (arrows indicate Purkinje cells); spinal cord; olfactory bulb; fourth ventricle; and liver. 3V, third ventricle; 4V, fourth ventricle; CA2, field CA2; DG, dentate gyrus; epl, external plexiform layer of the olfactory bulb; GL, granular layer; HiF, hippocampal fissure; igr, internal granular layer of the olfactory bulb; mdl, mediodorsal thalamic nucleus; ML, molecular layer; PCg, posterior cingulated cortex; PCp, the posterior part of the prepiriform cortex; PL, Purkinje cell layers; Pv, paraventricular thalamic nucleus; Str, the superior thalamic radiation; Te Aud, the auditory area of the temporal cortex.

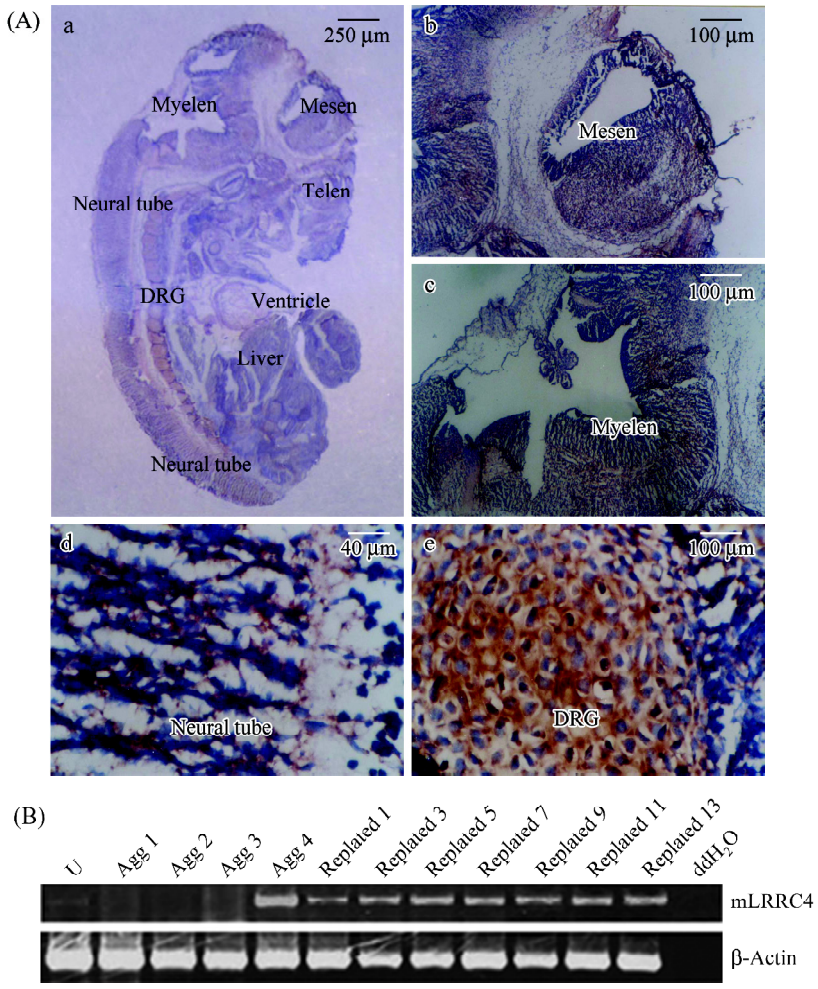


**Fig. 3 Expression profiles of murine leucine-rich repeat C4 protein (mLRRC4) in 12.5 d BALB/c mouse embryo (A) and during retinoic acid (RA)/P19 cell neuronal differentiation (B)**

(A) a, slit profiles of mLRRC4 in BALB/c mouse embryo; b–e, partially magnified image of plate a. DRG, dorsal root ganglia; Mesen, mesencephalon; Myelen, Myelencephalon; Telen, telencephalon. (B) P19 cell samples were prepared from cell lysates of non-induced wild-type P19 cells, and from aggregated RA-induced wild-type P19 cells for 1 d (Agg1), 2 d (Agg2), 3 d (Agg3), and 4 d (Agg4), and from neuronal differentiated RA/P19 cells for 1 d (Replated1), 3 d (Replated3), 5 d (Replated5), 7 d (Replated7), 9 d (Replated 9), 11 d (Replated11), and 13 d (Replated13). β-Actin was used as an internal control. ddH2O, double distilled water; U, uninduced wild-type P19 cells.

detected with sense probes.

P19 cells are known to differentiate into neuronal cells by both RA stimulation and cellular aggregation [13]. As murine embryonic carcinoma cell line P19 is a good model system for *in vitro* study of the molecular mechanism of mammalian neuronal differentiation [14], we were kindly given the cDNA of RA-induced neuronal differentiation of embryonic carcinoma P19 cells by Dr. Naihe JING [15, 16]. RT-PCR showed that mLRRC4 mRNA was barely detectable in undifferentiated P19 cells, and its expression was detected at the end of the induction phase (agg4) and at the relatively stable differentiation stage (Replated 1, 3, 5, 7, 9, 11, 13) [**Fig. 3(B)**]. These results implied a possible role for mLRRC4 in neuronal development and differentiation.

## LRRC4 promotes neurite extension of hippocampal neurons

Extracellular proteins containing both LRRs and Ig domains have been shown to participate in axon guidance [1]. We used a neurite outgrowth assay [17] to measure the ability of LRRC4 to promote neurite outgrowth. Hippocampal neurons from 18.5 d-old mouse embryos were dissected, dissociated, and plated at low density onto confluent monolayers of U251 cells expressing either LRRC4 or vector (control). Forty-eight hours after plating,

cells were fixed and stained with anti- microtubule associated protein-2 antibody to visualize the neurites. Representative photomicrographs of these cultures show an increase in neurite outgrowth from neurons cultured on U251 cells expressing LRRC4 [**Fig. 4(A,B)**]. To quantitate this effect, the length of the longest neurite per neuron, which did not have contacts with nearby neurons and was at least twice the length of the widest cell body diameter, was measured. Over 100 neurons for each condition were measured in three independent experiments [**Fig. 4(C)**]. Neurons grown for 48 h on U251 cells expressing LRRC4 had neurites with an average length of 70.7±14.3 μm, whereas neurites from neurons grown on control U251/vector cells were 48.1±7.9 μm in length ($P$ <0.001; ANOVA). These results show that LRRC4 promotes neurite outgrowth.

**LRRC4 induces glioblastoma U251 cell differentiation**

The stable transfectants U251/LRRC4 and U251/vector [U251/pcDNA3.1(+)-LRRC4 and U251/pcDNA3.1(+)] [10] were cultured in DMEM and collected for further detection. Astrocytic differentiation of cells was analyzed by Western blot [**Fig. 5(A)**] and indirect immunofluorescence [**Fig. 5(B)**] using anti-GFAP antibody. U251/vector cells showed a low level expression of GFAP, however, the expression of GFAP in U251/LRRC4 cells was more abundant. There were no differences in the levels of α-tubulin or 4′,6′-diamidino-2-phenylindole between the cell groups.

## Discussion

We have identified a series of novel LRPs: mLRRC4 (GenBank accession No. AF290542 or DQ199325); rLRRC4 (GenBank accession No. DQ119102); bLRRC4 (GenBank accession No. DQ164537); and hLRRC4 (GenBank accession No. AF196976). These four proteins show clear homology with each other; they all contain nine and a half LRRs flanking amino- and carboxy-flanking region and one IgC2 domain close to the transmembrane region, and their domain organizations are highly similar (**Fig. 1**). This domain relationship suggests a common evolutionary origin.

The previous research indicated that LRRC4 is highly expressed especially in the brain [12]. In this study, we used ISH to further examine the specific distribution of LRRC4 in brain tissue. The expression of mLRRC4 could be detected all over the central nervous system with the strongest expression in the granule cells of hippocampal CA1 and CA2, and the Purkinje cell layers in the developing mouse embryo [**Fig. 3(A)**] and post-natal mouse (**Fig. 2**), suggesting that the LRRC4 gene is involved in the normal function of the nervous system.

There are an increasing number of published reports on mammalian LRPs having both LRR and Ig domains, and some of these LRPs were reported to be involved in nervous system development, neuron and glia cell differentiation and the promotion of neurite growth, such as AMIGO [1], slit [18,19], NGL-1 [2] and NGL-2 [20]. The P19 cell line, derived from a teratocarcinoma induced in C3H/HC mice, is one of the most extensively studied, pluripotent embryonal carcinoma cell lines [13,21]. After treatment with RA and aggregation, P19 stem cells differentiate along a neural pathway, forming both neuron-like cells (on the fourth day of differentiation) and glia-like cells (on the seventh day of differentiation) [15,16]. RT-PCR showed different expression profiles of mLRRC4 mRNA in a P19 cell differential model. Expression of mLRRC4 was detected at the end of the induction phase and was relatively stable at the replated stage [**Fig. 3(B)**], which implied a possible role for mLRRC4 at earlier stages of neuronal development and differentiation. LRRC4 participates in the differentiation of neuron and glia cells [**Fig. 3(B)**] and

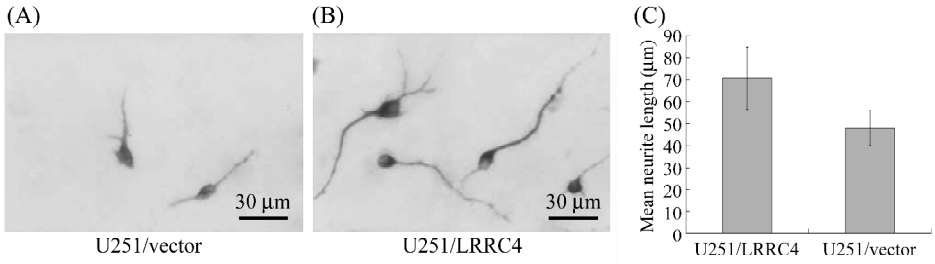


**Fig. 4 Leucine-rich repeat C4 protein LRRC4 promoted neurite outgrowth from primary cultured hippocampal neurons**

Representative image of hippocampal neurons of mouse embryos at day 18.5 plated on the control U251/vector (A) and U251/LRRC4 (B) cells. Only the extensions, which were twice the length of the cell soma, were counted. (C) Neurite length of at least 100 neurons for each condition in three independent experiments is presented as the mean neurite length. *$P$<0.001, $t$-test.

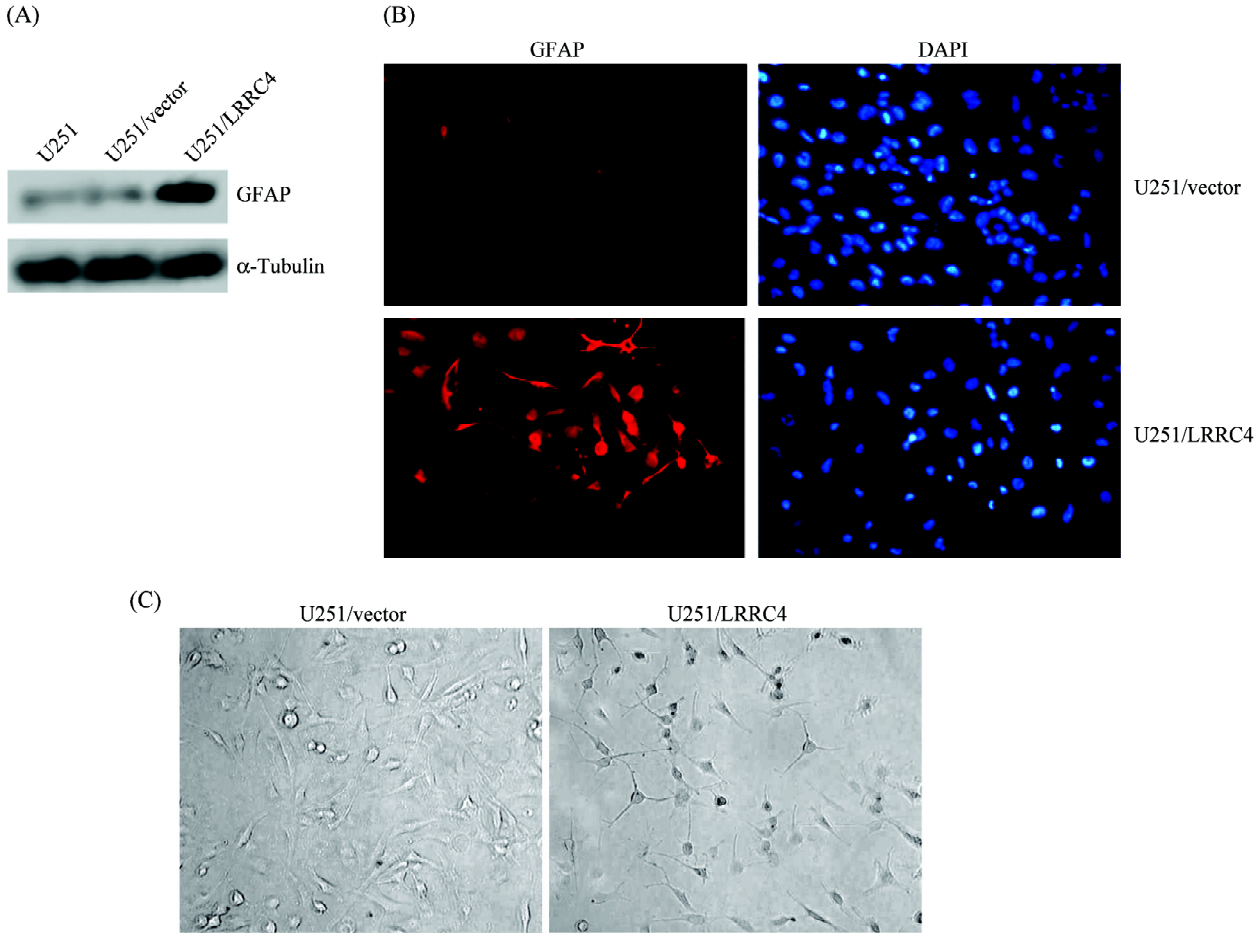


**Fig. 5 Leucine-rich repeat C4 protein LRRC4 induces differentiation of glioblastoma U251 cells into astrocyte-like cells**

(A) Western blot analysis of changes in glial fibrillary acidic protein (GFAP) expression in U251/vector and U251/LRRC4 transfectants. The expression of α-tubulin was viewed for an internal protein control. (B) Changes of GFAP expression in U251/vector and U251/LRRC4 transfectants detected by indirect immunofluorescence (10×). 4',6'-Diamidino-2-phenylindole (DAPI) nucleus staining was viewed as an internal control. (C) Morphological changes of U251/vector and U251/LRRC4 transfectants under an inverse light microscope (10×).

promotes neurite outgrowth (**Fig. 4**).

LRRC4s are essential nervous system-specific proteins. The re-expression of LRRC4 has the potential to suppress tumorigenesis *in vivo* and cell proliferation and invasion of U251 cells *in vitro* [22], and LRRC4 is a potential glioma suppressive gene [10,11,22,23]. Although U251 cells are blocked in $G_1$ late phase, transfection of exogenous LRRC4 does not induce apoptosis in U251 cells, therefore, LRRC4 might induce U251 cell differentiation. The expression of GFAP is a defining characteristic of astrocytes [24], and GFAP content and the elongation of cellular processes indicates the magnitude of astrocytic differentiation in glioma cells [25]. In this study, astrocytic differentiation of glioma cells was analyzed by expression of GFAP. The transfectant with exogenous LRRC4 showed the differentiated phenotype, in contrast to the parent cells (**Fig. 5**). The expression of GFAP in transfectant with LRRC4 was evidently increased compared to the transfectant with vector. Differentiating cells manifest a prolonged $G_1$ phase and inhibition of $G_1$ to S phase transition [26,27]. Therefore, LRRC4 modulates the ERK/AKT/NF-κB and JNK2/p-c-Jun/mp53 signaling pathways to block U251 cells in the $G_1$ late phase, inhibits U251 cell proliferation [10,11,23], and induces U251 cells to differentiate into astrocytes-like cells.

Taken together, the data suggests that LRRC4, as a suppressive gene, is involved in normal development and differentiation of the nervous system, and tumorigenesis of glioma. The re-expression of LRRC4 in U251 cells could inhibit glioma cell proliferation and induce glioma cells to

differentiate into astrocytes.

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