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LRRC4 Inhibits Human Glioblastoma Cells Proliferation, Invasion, and proMMP-2 Activation by Reducing SDF-1α/CXCR4-Mediated ERK1/2 and Akt Signaling Pathways

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Abstract Gliomas take a number of different genetic routes in the progression to glioblastoma multiforme, a highly invasive variant that is mostly unresponsive to current therapies. The α-chemokine stromal cell-derived factor (SDF)-1α binds to the seven transmembrane G-protein-coupled CXCR-4 receptor and acts to modulate cell migration and proliferation by activating multiple signal transduction pathways. Leucine-rich repeats containing 4 (LRRC4), a putative glioma suppressive gene, inhibits glioblastoma cells tumorigenesis in vivo and cell proliferation and invasion in vitro. We also previously demonstrated that LRRC4 controlled glioblastoma cells proliferation by ERK/AKT/NF-κB signaling pathway. In the present study, we demonstrate that CXC chemokine receptor 4 (CXCR4) is expressed in human glioblastoma U251 cell line, and that SDF-1α increases the proliferation, chemotaxis, and invasion in $CXCR4^+$ glioblastoma U251 cells through the activation of ERK1/2 and Akt. The reintroduction of LRRC4 in U251 cells inhibits the expression of CXCR4 and SDF-1α/CXCR4 axis-mediated downstream intracellular pathways such as ERK1/2 and Akt leading to proliferate, chemotactic and invasive effects. Furthermore, we provide evidence for proMMP-2 activation involvement in the SDF-1α/CXCR4 axis-mediated signaling pathway. LRRC4 significantly inhibits proMMP-2 activation by SDF-1α/CXCR4 axis-mediated ERK1/2 and Akt signaling pathway. Collectively, these results suggest a possible important ''cross-talk'' between LRRC4 and SDF-1α/CXCR4 axis-mediated intracellular pathways that can link signals of cell proliferation, chemotaxis and invasion in glioblastoma, and may represent a new target for development of new therapeutic strategies in glioma. J. Cell. Biochem. 103: 245–255, 2008. © 2007 Wiley-Liss, Inc.

Key words: glioblastoma; LRRC4; SDF-1α/CXCR4; chemotaxis; invasion; MMP-2; ERK/Akt

Glioblastomas are the most common and lethal human primary brain tumors, and are characterized by high invasiveness, neoangiogenesis, and extended necrosis. The transformation of normal cells into gliomas occurs as a result of the stepwise accumulation of a series of genetic lesions [Maher et al., 2001]. Leucine-rich repeats containing 4 (LRRC4) is a potential glioma suppressive gene [Wu et al., 2006a]. The conspicuous absence of LRRC4 in high-grade gliomas directly contributes to increasing tumor grade [Wu et al., 2006a]. The re-expression of *LRRC4* can significantly suppress glioblastomas U251 cells tumorigenesis in vivo and cell proliferation and invasion in vitro [Zhang et al., 2005a; Wu et al., 2006a,b].

Minghua Wu, Qiong Chen, and Dan Li contributed equally to this work.

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Stromal cell-derived factor (SDF)-1 (recently renamed CXCL12) and its CXC chemokine receptor 4 (CXCR4) are involved in normal and malignant glial cell proliferation and migration [Barbero et al., 2002; Hong et al., 2006]. SDF-1 exerts its activity by interacting with the CXCR4 receptor, a member of the G protein-coupled receptor superfamily. CXCR4, similar to SDF-1, is expressed in various tissues and also at brain level in different cell types, including endothelial cells, embryonic germinal neuroepithelium, and mature neurons, glial cells, and microglia cells, and seems to be involved in different CNS pathologies [Bajetto et al., 2001]. SDF-1 and CXCR4 are constitutively highly expressed in several human cancer tissues and cell lines such as human melanoma, gliomas, pancreatic cancer, colon cancer, and malignant ovarian cancer [Barbero et al., 2002; Guleng et al., 2005; Porcile et al., 2005; Scala et al., 2006; Wehler et al., 2006]. Recent data showed that CXCR4 and SDF-1 mRNAs are co-localized in glioblastomas and that their expression increase with tumor grade and is associated with regions of necrosis and angiogenesis [Rempel et al., 2000].

Tumor cell invasion and metastasis are regarded as multistep phenomena involving the proteolytic degradation of the basement membrane (BM) and extracellular matrix (ECM), physical movement of tumor cells. Among the many steps in invasion and metastasis, excessive degradation of the matrix is one of the hallmarks of this process [Chintala et al., 1999]. Many proteinases are capable of degrading ECM components, but two families of enzymes that appear to be particularly important for ECM degradation, namely, the matrix metalloproteinases (MMPs (MMP-2 and MMP-9)) and urokinase-type plasminogen activator (uPA). These proteinases have been closely linked with the invasive and metastatic phenotype of cancer cells [Nakada et al., 2003]. In this work, we studied the effect of the re-expression of LRRC4 on glioblastoma U251 cells chemotaxis, invasion, and secretion of MMP-2 and MMP9 by SDF-1α/CXCR4-mediated ERK1/2 and Akt signaling pathways.

MATERIALS AND METHODS

Reagents and Materials

Anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-Akt (Ser473), and anti-Akt were purchased from Cell Signaling Technology (Beverly, MA). Anti-CXCR4 antibody was purchased from Neomarkers (Fremont, CA). Human-SDF-1α was purchased from Pepro Tech EC Ltd. Pines Biolabs (London, UK). PD98059 was purchased from Upstate (NY). Ly294002 was purchased from Cell Signaling Technology.

Generation of High Specific Antibody to hLRRC4

An appropriate nucleic acid sequence was chose by analyzing of the hydrophilicity, antigenicity, and transmembrane domain of the LRRC4 protein. The appropriate sequence that did not contain transmembrane domain was amplified by PCR, and subcloned into a prokaryotic expression vector pGEX-4T-2 to construct a recombinant. *E. coli* JM109 transformed with the recombinant was induced by IPTG to express GST-fusion protein, and it was found that the fusion protein expressed as insoluble inclusion bodies. Then the purified inclusion bodies obtained by washing repeatedly with 0.2% deoxycholic acid and 1 M urea were used to immunize rabbits. Once the titer of antiserum reaches to $1{:}10^8$ by indirect ELISA, collect and purify the serum.

Tissues Collection and Immunohistochemistry Stain

The human embryo tissue specimens and glioma specimens were obtained from at the pathology department of Xiangya Hospital, Hunan, P.R. China, and were graded according to the revised WHO classification [Kleihues et al., 1993]. All specimens were routinely fixed in 4% paraformaldehyde at 4°C and embedded in paraffin. Sections (5 μm) cut from the tissue blocks were dewaxed in xylene and then rehydrated. The endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min at room temperature. Antigen unmasking with heat retrieval in citrate buffer (pH 6.5) was accomplished by placing the slides in a microwave pressure cooker (1,500 W at power level 6) for 30 min (or antigen retrieval was accomplished by pressure cooking for 25 min in citrate buffer). Slides were then incubated with normal goat serum for 30 min at room temperature, followed by incubation rabbit anti-human LRRC4 antibody diluted 1:2,000 in 1% rabbit serum/PBS" for 90 min at room temperature. The slides were washed again in PBS and then incubated for 30 min with an avidin and

biotinylated peroxidase complex (Vectastain; Vector Laboratories). Peroxidase activity was developed by a solution of 5 mg of 3-amino-9-ethylcarbazole (AEC) (Sigma–Aldrich) at room temperature under microscopic control. Finally, the sections were washed in double-distilled water, counterstained, and the immunoreactivity was assessed under the light microscope.

Cells and Transfectants

Cells were cultured at 37°C in 5% CO_2 in DMEM (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin, 100 g/ml streptomycin, and 1% nonessential amino acids. pcDNA3.1 (+) (Invitrogen, Carlsbad, CA) and pcDNA3.1 (+)-LRRC4 expression vectors were used for stable transfection. At 24 h post-transfection, cells were split at a 1:3 dilution and cells were challenged with 500 μg/ml G418 (Geneticin sulfate; Invitrogen) and left for 2–3 weeks with change of medium every 3 days, and formed a stable transfected clone pool. Western blot and cell immunochemistry were used to identify stable transfectants.

RT-PCR

Total RNA was extracted according to the protocol of TRIZOL Reagent (Invitrogen) and then treated with DNase I to eliminate possible contaminated genomic DNA. The procedure of reverse transcription followed the instructions of Promega (Madison, WI). The gene-specific primers used for CXCR4 was as follows: CXCR4, 5′-AATCTTCCTGCCCACCA-TCT-3′ (sense) and 5′-GACGCCAACATAGAC-CACCT-3′ (antisense); the primers for GAPDH was 5′-CCACCATGGGCAAATTCCATGGCA-3′ (sense) and 5′-TCTAGACGGCAGGTCAGGTC-CACC-3′ (antisense). The primers for LRRC4 have been reported previously [Wu et al., 2006a].

Western Blot

Western bolt was performed as reported previously [Wu et al., 2006a]. Briefly, cells were lysed in 1% NP40, 20 mM Tris-HCl (pH 8), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM sodium orthovanadate, and 10 mM NaF for 20–30 min at 4°C. Cell lysates were assayed for protein content using the BCA protein assay kit (HyClone, Beijing, China). Proteins (50 μg) were separated by SDS–PAGE and blotted with primary antibodies. Anti-GAPDH antibody (Santa Cruz Biotechnology) was used as a loading control.

FACS Analysis

The expression of CXCR4 was evaluated by fluorescence-activated cell sorting (FACS) analysis as previously described [Libura et al., 2002]. The CXCR4 antigen was detected with phycoerythrin (PE)—anti-CXCR4 monoclonal antibody. Briefly, the cells were stained in phosphate-buffered saline (PBS; Ca- and Mg-free) supplemented with 5% bovine calf serum. After the final wash, cells were fixed in 1% paraformaldehyde prior to FACS analysis, performed using the FACscan (Becton Dickinson, San Jose, CA).

Stimulation of Cells

Cells were washed twice with DMEM (Life Technologies, Inc.) and resuspended in the same medium. Cells were starved 24 h at 37°C and then stimulated with different concentrations of SDF-1α at 37°C for various periods. After stimulation, cells were used to experiment as described below.

Cell Proliferation by MTT Assay

The MTT assay was performed according to the manufacturer's recommendations (Promega). Briefly, cells were seeded in 96-well plates at 5×104/well in 100 μl of DMEM medium containing 0.5% bovine serum albumin (BSA), 2% BCS, or 10% BCS plus various concentrations of SDF-1α (0, 1, 10, and 100 ng/ml). After 72 h, 20 μl of Cell Titer 96 Aqueous One Solution reagent were added to each well and plates were incubated for 3–4 h. Subsequently, plates were read at 570 nm using an automated plate-reader.

Chemotaxis Assay

The chemotaxis assay was performed in 96-well plates containing 5 μm porosity inserts (Costar Corp., Kennebunk, ME). Cells grown in DMEM medium containing 10% FCS were washed twice and suspended as 10×10^6 cells per ml in DMEM medium containing 0.5% BSA. Chemokines were then added to the bottom wells, and 100 ml (1×10^6) of cells were loaded onto the inserts. Cells migrating to the bottom well were collected after 4 h. Five random fields per filter at 40× magnification were counted to quantitate cell migration counts.

Cell Invasion Assay

Cell invasion was measured by a Matrigel invasion chamber assay, which was performed using 6.5-, 8-mm pore size Transwell chambers (Corning, Corning, NY) [Wu et al., 2006b]. Matrigel (Becton Dickinson) was diluted in cold distilled water (200 mg/ml), 0.1 ml added to the upper well of the Transwell chamber, and dried in a sterilehood. The Matrigel was reconstituted with medium for 1 h at 37°C before cells being added. Cells were starved 24 h in serum free medium and resuspended at a concentration of 2.5×10^5 cells/ml in serum-free medium containing 0.1% BSA. Cell suspension (0.2 ml) was added to the top of each well, and a 10 mg/ml fibronectin solution was added to the bottom well of the chamber as a chemoattractant. After 36 h, the cells remained in the top chamber were removed from the upper surface of the filters using a cotton swab. The cells migrated to the lower surface of the filter were fixed with methanol, stained with H&E. Five random fields per filter at 40× magnification were counted to quantitate cell migration. The data were presented as the mean value of cells per high-power field in triplicate from two independent experiments.

Gelatin Zymography

To determine the relative concentration and activity of gelatinases in the conditioned medium, SDS–PAGE gelatin zymography was performed by using a previously reported method [Koshiba et al., 1997]. Briefly, 40 µl of each conditioned medium was treated with SDS–PAGE sample buffer without boiling and reduction. Samples were fractionated in a 10% polyacrylamide gel by electrophoresis at 100 V for 90 min at 4°C. The gels were soaked in 0.25% Triton X-100 for 30 min at RT, to remove the SDS, and incubated in a development buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM $CaCl_2$) containing 5 mM phenylmethylsulfonyl fluoride, a serine protease inhibitor, at 37°C overnight, to allow proteinase digestion of the substrate, and then stained with 0.25% Coomassie blue R-250 in 40% isopropanol for 2 h and destained with 7% acetic acid. Gelatinolytic activities appeared as clear bands of digested gelatin against a dark blue background of stained gelatin.

Statistical Analysis

Experiments were performed and repeated at least three times. Data are expressed as mean ± SE, and statistical significance was assessed by Student's *t*-test for independent groups using the SPSS 11.0 program. $P \leq 0.05$ was considered statistically significant.

RESULTS

Expression Distribution of LRRC4 Protein in Human Embryo, Glioma Tissue, and U251 Cells Transfected With LRRC4 or Mock

We used the anti-LRRC4 rabbit polyclone antibody (described in detail under Materials and Methods Section) to further detect the expression of hLRRC4 in various embryo tissues by immunohistochemistry stain. Consistent with the expression pattern of mLRRC4 and hLRRC4 that had been detected by Northern-blot and RT-PCR [Zhang et al., 2005a], we found that hLRRC4 specially expressed in brain, and did not express in heart, liver, lung, kidney, oesophagus, small intestine, testicle, thymus, pancreas, adrenal gland, bladder, and skin. As shown in Figure 1, we also detected the hLRRC4 expression in astrocytes from normal brain cortex and different WHO grade gliomas with hLRRC4 antibody and performed a quantification assay by gray scanning. We found that cells counts of astrocytes from normal brain cortex were low, but expression of hLRRC4 was very strong. The cells counts of astrocytes were increasing along with the WHO grade increase of gliomas, but the expression intensity of hLRRC4 was gradually decreased even completely absent in WHO grade IV gliomas (data not shown). Also, the nuclear expression of hLRRC4 protein was observed in the astrocytes in normal brain and different grade glioma tissues.

Plasmids pcDNA3.1(+)/LRRC4 containing full length LRRC4 cDNA and the pcDNA3.1(+) vector (mock) were transfected into human glioblastoma U251 cells and pool clones were selected. Western blot and RT-PCR were then used to examine the LRRC4 protein expression level between U251/LRRC4 cells and U251/mock cells (unpublished work).

LRRC4 Inhibits U251 Cells Proliferation, Chemotaxis, and Invasion Through Reducing SDF-1α/CXCR4 Axis

Cells were serum starved for 24 h and treated with increasing concentrations of SDF-1α

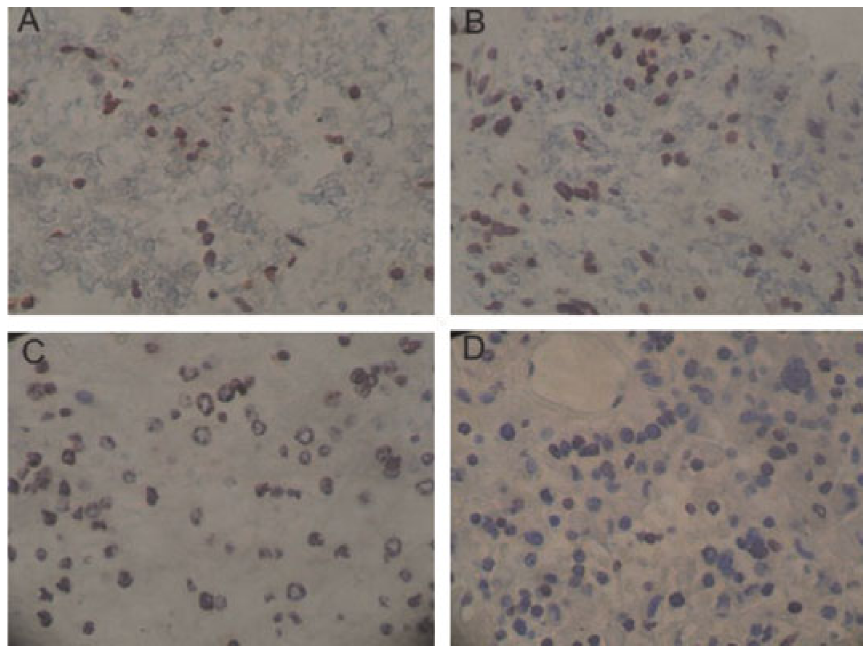

Fig. 1. Expression of hLRRC4 in astrocytes from normal brain cortex and different grade gliomas by immunohistochemistry stain with anti-rabbit hLRRC4 antibody at 1:2,000 dilution (40×, AEC staining). **A**: Astrocytes from the normal brain cortex. **B**: The WHO grade I astrocytoma. **C**: The WHO grade II astrocytoma. **D**: The WHO grade III anaplastic astrocytoma. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

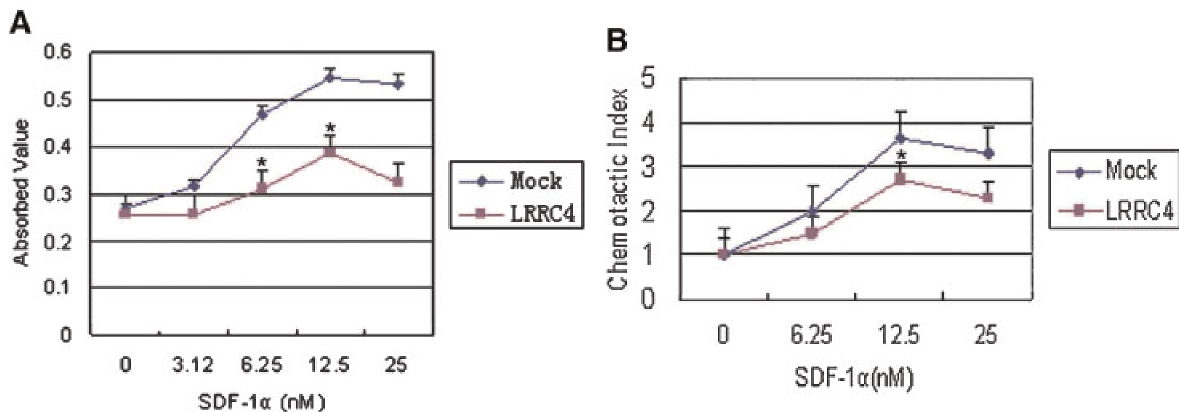

Fig. 2. LRRC4 inhibits U251 cells proliferation, chemotaxis, and invasion through SDF-1α/CXCR4 axis. **A**: Effect of LRRC4 on SDF-1α-induced proliferation in U251 cells. Mock/U251 and LRRC4/U251 cells were treated for 24 h with different concentrations of SDF-1α, and absorbed value of MTT was assessed. Values are the mean ± SE of six determinations from three independent experiments. Significantly different versus mock control values: $*P < 0.05$. **B**: Effect of LRRC4 on SDF-1α-induced chemotaxis in U251 cells. Mock/U251 and LRRC4/U251 cells were treated for 4 h with different concentrations of SDF-1α, and chemotactic index was assessed. Values are the mean ± SE of six determinations from three independent experiments. Significantly different versus mock control values: $*P < 0.05$. **C** Effect of LRRC4 on SDF-1α-induced invasion in U251 cells. Mock/U251 and LRRC4/U251 cells were treated for 24 h with SDF-1α (12.5 nM), and migrated cells were counted. Values are the mean ± SE of three determinations from three independent experiments. Significantly different versus mock control values: $*P < 0.01$. **D**: Effects of LRRC4 on CXCR4 expression in U251 cells with or without SDF-1αstimulation by Western blot with anti-CXCR4 antibody. GAPDH was used as the internal control. **E**: Effects of LRRC4 on CXCR4 expression in U251 cells by FACS analysis with anti-CXCR4 antibody. IgG isotype was used as the internal control. Values are the mean ± SE from three independent experiments. Significantly different versus mock control values: $*P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

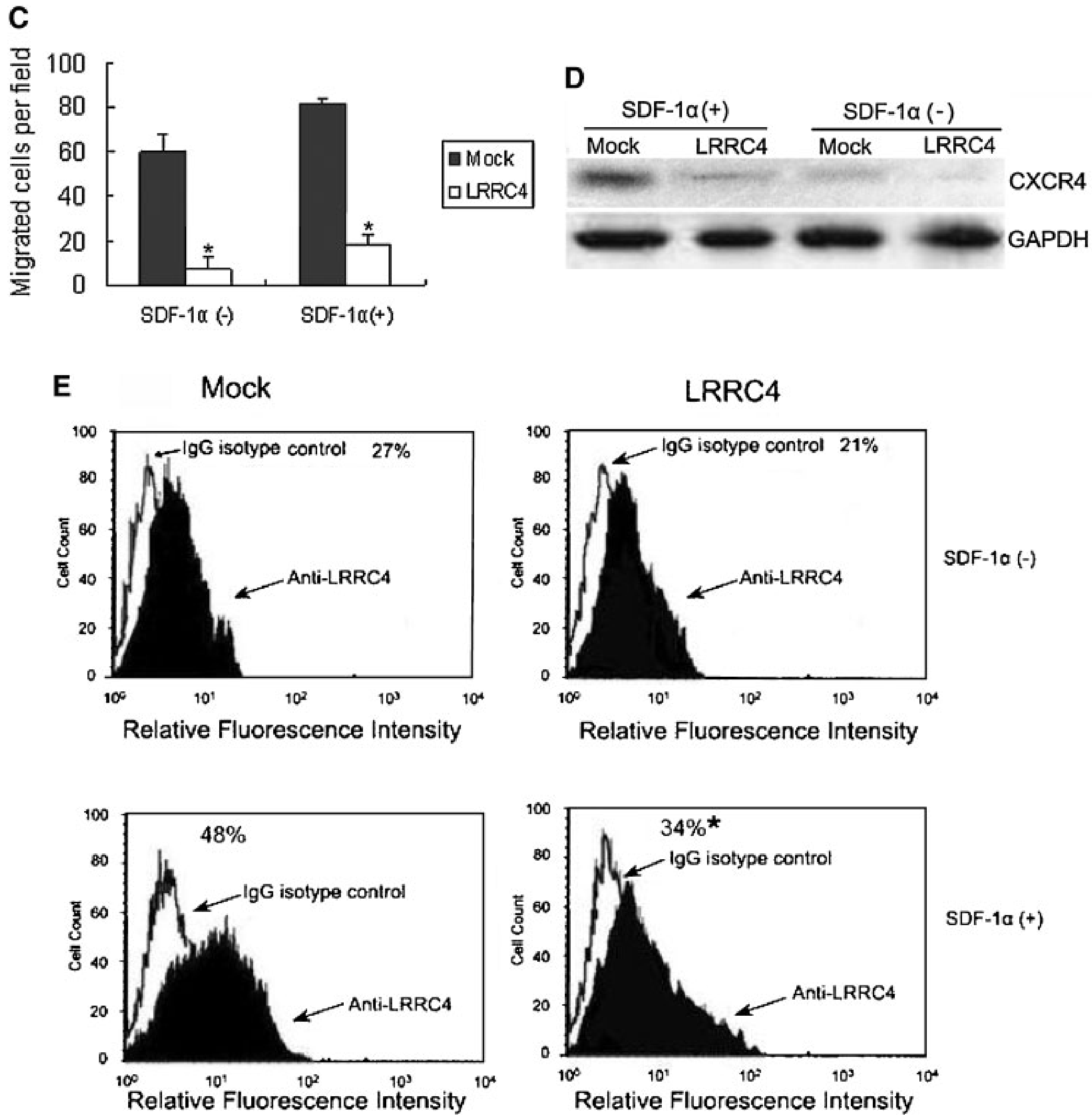

Fig. 2. (*Continued*)

(3.12–25 nM) for 24 h. The results, shown in Figure 2A, indicate that in both cell lines, SDF-1α caused a dose-dependent induction of proliferation with a maximum effect at 12.5 nM. Using higher concentrations of SDF-1α (25 mM), a downregulation of the response was observed, which is probably due to CXCR4 downregulation [Koshiba et al., 1997]. LRRC4 could inhibit the SDF-1α-induced proliferation in U251/LRRC4 cells, compared with that in U251/mock cells ($P < 0.01$).

We further analyzed the effect of LRRC4 on U251 cells' chemotaxis by exogenous SDF-1α stimulation. As shown in Figure 2B, SDF-1α also caused a dose-dependent induction of chemotaxis with a maximum effect at 12.5 nM in both U251/LRRC4 and U251/mock cells. Using higher concentrations of SDF-1α (25 mM), a downregulation of the response was observed. LRRC4 also could inhibit SDF-1α-induced the cells chemotaxis in U251/LRRC4 cells, compared with that in U251/ mock cells ($P < 0.01$).

Gliomas, a type of devastating primary brain tumors, are distinct from other solid, non-neural primary neoplasms, in that they display

extensive infiltrative invasive behavior but seldom metastasize to distant organs. To assess the invasion potential of the U251 cells following treatment with SDF-1α, we performed Matrigel matrix invasion assays (Fig. 2C). U251 cells transfected with mock migrate through these substrates, whereas U251 cells expressing LRRC4 do not. When U251/mock cells were treated with SDF-1α, they were able to migrate freely through the Matrigel matrix membrane. In contrast, when U251/LRRC4 cells were treated with SDF-1α, migration ability of U251 cells evidently decreased. LRRC4 could inhibit SDF-1α-induced U251 cells invasion.

The interaction between CXCR4 and SDF-1α appears to be unique, and SDF-1α exerts its activity by interacting with the CXCR4 receptor [Koshiba et al., 2003]. To analyze whether LRRC4 exerts its suppressive effect by reducing expression of CXCR4, the expression of the chemokine receptor CXCR4 in U251 cells, which were transfected with LRRC4 or mock, was studied. As shown in Figure 2D, we detected the CXCR4 expression in U251/mock, LRRC4 almost blocked completely the CXCR4 expression under basal conditions by Western blotting analysis. The exogenous SDF-1α evidently stimulated the expression of CXCR4 in U251/mock cells; however, the lower CXCR4 expression was detected in U251/LRRC4 cells after SDF-1α stimulation. The similar expression change of CXCR4 was observed by FACS analysis under basal conditions or after SDF-1α stimulation (Fig. 2E). The data suggested that U251 is a CXCR4^{+} cell line and LRRC4 exerts its suppressive effect through reducing SDF-1α/CXCR4 axis.

Effect of LRRC4 on ERK1/2 and AKT Signaling Pathway With or Without SDF-1α Stimulation

The previous research has reported that SDF-1α binds to CXCR4 receptor, activates multiple signal transduction pathways such as ERK/MAPK and PI-3K/AKT, and acts to modulate cell migration and proliferation [Zhang et al., 2005b]. By Western blot, we analyzed SDF-1α-induced ERK1/2 activation in both U251 cells transfected with LRRC4 or mock. Cells were treated with SDF-1α (12.5 nM), and the cell lysates were analyzed for the presence of phosphoryed, and thus activated, forms of ERK1/2. In both cell types, a significant amount of phosphorylated ERK1/2 was detected under basal conditions, even after 24 h of serum deprivation (Fig. 3A). In U251/mock cells, SDF-1α-induced activation of ERK1/2 was clearly evident after 5 min of stimulation, further increased after 15 min, and lasted up to 30 min (Fig. 3A). Similarly, in U251/LRRC4 cells, SDF-1α-induced ERK1/2 activation was detectable after 15 min of treatment and lasted up to 30 min (Fig. 3A), but phosphorylated level of ERK1/2 in all lanes was evidently lower than that in U251/mock. LRRC4 significantly inhibited the phosphorylated expression of ERK1/2 with or without SDF-1αstimulation (Fig. 3A). The analysis of cell lysates for the total expression of ERK1/2 ensured the equal loading of proteins in the different lanes (Fig. 3A). Cells were starved for 24 h with SDF-1α (12.5 nM) in the presence of PD98059 (10 μM), a pharmacological inhibitor of MAPK/ERK kinase (MEK). PD98059 reduced both basal (2) and SDF-1α-stimulated ERK1/2 phosphorylation in both cell lines. LRRC4 synergizes the inhibitory effect of PD98059 on phosphorylated expression of ERK (Fig. 3A).

Another important signal transduction pathway in glioblastoma is the PI3K/Akt pathway. It was shown that besides the ERK1/2 pathway, SDF-1α/CXCR4 activates PI3K/Akt pathway [Ganju et al., 1998; Barbero et al., 2003]. Thus, we examined the role of LRRC4 in SDF-1α/CXCR4-induced activity of Akt by Western blot analysis. U251/mock or U251/LRRC4 cells were challenged with SDF-1α (12.5 nM), and their lysates were analyzed for Akt phosphorylation using an antibody that reacts specifically with the phosphorylated Ser473 located at the COOH terminus. As shown in Figure 3B, our results demonstrated that in the U251/mock cells, Akt activation had already occurred under basal conditions and was further increased after 15–30 min of SDF-1α stimulation. Similar trend was obtained in U251/LRRC4 cells, but phosphorylated level of AKT in all lanes was evidently lower than that in U251/mock, indicating that LRRC4 significantly inhibits the phosphorylated expression of AKT with or without SDF-1α stimulation (Fig. 3B). The same lysates were also analyzed for total expression of Akt to ensure an equal loading of proteins in the different lanes. To analyze the involvement of Akt in the LRRC4 suppressive activity mediated by SDF-1α/CXCR4, we studied the effect of LY294002, a specific PI3K inhibitor, on SDF-1α-dependent Akt activation. As shown in Figure 3B, this compound strongly reduced

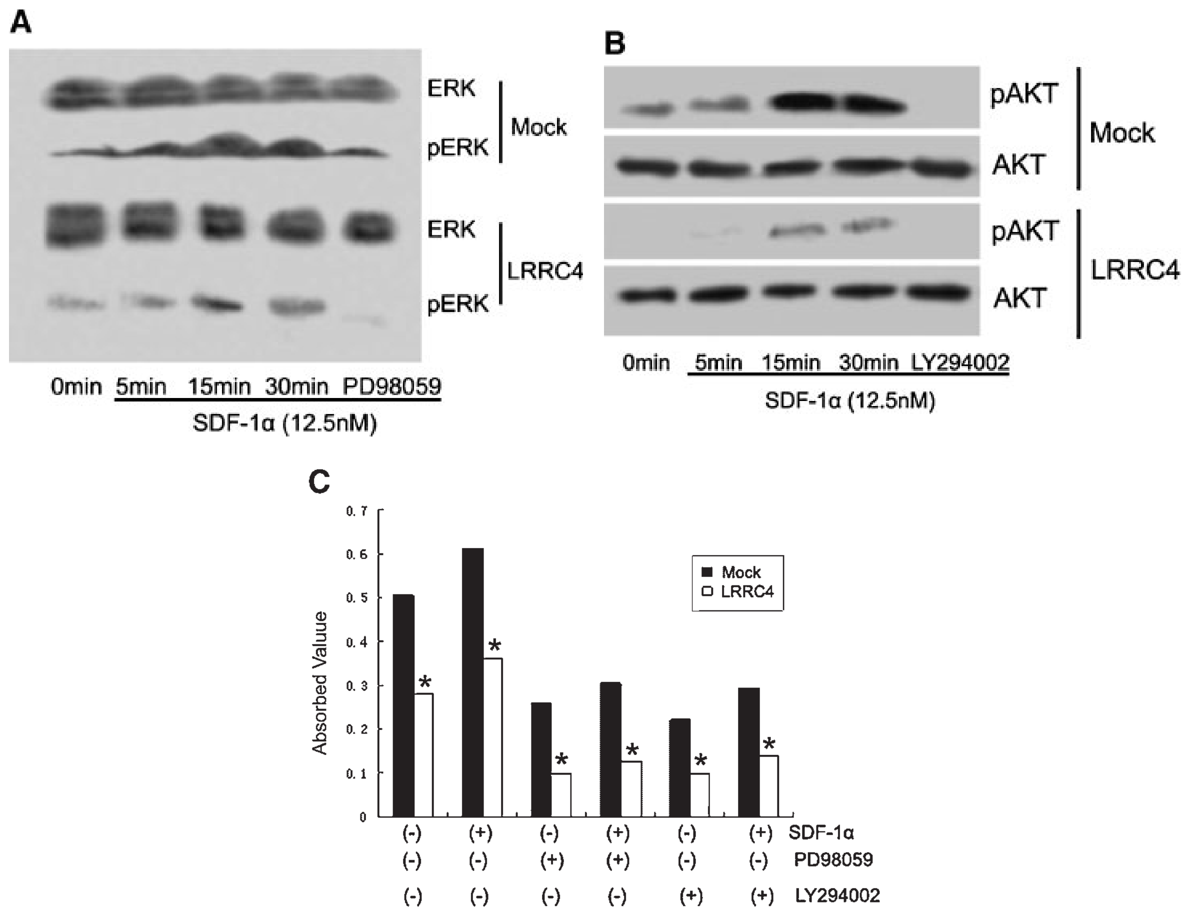

Fig. 3. **A**: Effect of LRRC4 on SDF-1α-induced ERK1/2 phosphorylation in U251 cells by Western bolt analysis. Mock/U251 and LRRC4/U251 cells were starved for 24 h before SDF-1α (12.5 nM) stimulation, and cells were treated with SDF-1α for different time. About effects of PD98059 on SDF-1α-induced pERK1/2, PD98059 (10 μm) was present for 10 min before SDF-1α (12.5 nM) stimulation for 15 min. The analysis of cell lysates for the total expression of ERK1/2 ensured the equal loading of proteins in the different lanes. **B**: Effect of LRRC4 on SDF-1α-induced AKT phosphorylation in U251 cells by Western bolt analysis. Mock/U251 and LRRC4/U251 cells were starved for 24 h before SDF-1α (12.5 nM) stimulation, cells were treated with SDF-1α for different time. About effects of LY294002 on SDF-1α-induced pAKT, LY294002 (10 μm) was present for 30 min before SDF-1α (12.5 nM) stimulation for 15 min. The analysis of cell lysates for the total expression of AKT ensured the equal loading of proteins in the different lanes. **C**: Effect of PD98059 or LY294002 on SDF-1α-induced proliferation in mock/U251 and LRRC4/U251 cells. When indicated, PD98059 (10 μM) was added to the cells for 10 min before SDF-1α (12.5 nM) stimulation and during the chemokine stimulation; LY294002 (10 μM) was added to the cells 30 min before chemokine stimulation, then, cells were induced for 24 h. Values are the mean ± SE of six determinations from three independent experiments. Significantly different versus mock control values: * $P < 0.05$.

AKT activation induced by SDF-1α treatment in U251/mock and U251/LRRC4 cells. LRRC4 synergized the inhibitory effect of LY294002 on phosphorylated expression of AKT (Fig. 3B). Moreover, either PD98059 or LY294002 inhibited basal level or SDF-1α-induced proliferation and invasion in both U251/mock and U251/LRRC4 cells (Fig. 3C).

Effect of LRRC4 on MMP-2 and -9 Secretions With or Without SDF-1αStimulation

Earlier reports had shown that expression and activation of MMPs gene products are coordinately controlled by the activation of various signal-related protein kinases, including ERK1/2 and Akt [Park et al., 2001; Zhang et al., 2004]. To determine whether LRRC4 controls matrix metalloproteinases production by SDF-1α/CXCR4-mediated ERK/MAPK and Akt signaling pathway, we analyzed the MMP-2 and-9 proteinases activity in these cells using gelatin zymography. As shown in Figure 4A, proMMP-2 (72 kDa) and -9 (92 kDa) activity did not change in U251/mock cells (pane 4–6) and U251/LRRC4 cells (pane 10–12), even after SDF-1α stimulation (pane 1–3, 7–9). LRRC4

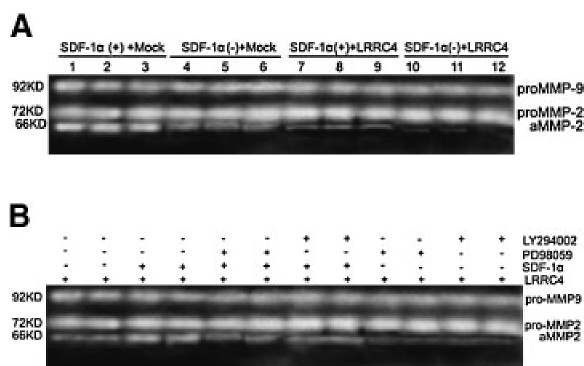

Fig. 4. **A**: Typical zymographic pattern of LRRC4 on matrix metalloproteinase with or without SDF-1α stimulation by gelatin zymography. proMMP-9 (92 kDa): latent form of matrix metalloproteinase (MMP-9); proMMP-2 (72 kDa): latent form of MMP-2; aMMP-2 (66 kDa): activated form of MMP-2. **B**: Effect of PD98059 or LY294002 on SDF-1α-induced MMP-2 activation in LRRC4/U251 cells and Mock/U251 cells (not shown). When indicated, PD98059 (10 μM) was added to the cells for 10 min before SDF-1α (12.5 nM) stimulation and during the chemokine stimulation; LY294002 (10 μM) was added to the cells 30 min before SDF-1α stimulation, and then cells were induced for 24 h.

could inhibit activation of proMMP-2 (66 kDa) with (lane 7–9) or without (lane 10–12) SDF-1α stimulation. However, activated form of MMP-9 (82 kDa) was not tested in all cell lines. Moreover, proMMP-2 activation was blocked by the PI3Kinhibitor LY294002 or MEK inhibitor PD98059 with or without SDF-1α stimulation (Fig. 4B).

DISCUSSION

LRRC4, a glioma suppressive gene, was cloned and characterized from human chromosome 7q31-32 using a computer-assisted positional cloning strategy combined 5′-RACE (rapid amplification of cDNA ends) in our laboratory [Wang et al., 2002]. *LRRC4* is predominantly expressed in the normal brain tissue, but is deleted or downregulated in primary brain tumor biopsies (up to 87.5% in gliomas) [Park et al., 2001; Libura et al., 2002]. In this study, we tested the expression distribution of LRRC4 in different grades of glioma tissues by immunohistochemistry stain with LRRC4 antibody, and found that expression of LRRC4 is gradually downregulated along with increasing glioma grade. The data further confirmed that the downregulated or absent expression of LRRC4 directly involved in glioma malignant progression, which has been tested by Northern-blot and RT-PCR [Wu et al., 2006a].

Glioblastoma multiforme is the most common malignant tumor of the adult central nervous system. The highly lethal nature of this tumor results from the acquisition of an invasive phenotype that allows the tumor cells to infiltrate surrounding brain tissue [Kunapuli et al., 2004]. The SDF-1/CXCR4 system plays an important role not only in promoting metastasis, but also in enhancing chemotaxis, invasion, and adhesive properties of cancer cells, including prostate cancer, rhabdomyosarcoma, breast cancer, human ovarian epithelial tumor, and glioma [Scotton et al., 2002; Taichman et al., 2002; Chen et al., 2003; Hong et al., 2006]. The over-expression of SDF-1α (in one-third glioblastoma) and its receptor CXCR4 (in all glioblastoma) correlates with the malignancy grade of the glioma, and foster glioma cells migration, proliferation, and survival [Rempel et al., 2000; Barbero et al., 2002, 2003; Zhou et al., 2002]. Both LRRC4 and inhibitor of CXCR4 receptor blocks glioma cells proliferation and invasion [Rubin et al., 2003; Zhang et al., 2005a; Hong et al., 2006; Redjal et al., 2006; Wu et al., 2006a]. The absent expression of LRRC4 (Fig. 1) as well as over-expression of CXCR4 [Rempel et al., 2000; Barbero et al., 2003] contributes to grade IV glioblastoma. We, therefore, investigated the effects of re-expressed LRRC4 on CXCR4 expression and the corresponding proliferation, chemotaxis, and invasion properties of a human U251 glioblastoma cell line, in which LRRC4 is absent [Wu et al., 2006a]. Our data suggested that the re-expressed LRRC4 significantly inhibited SDF-1α-induced U251 cells proliferation, chemotaxis and invasion by reducing the expression of CXCR4 (Fig. 2).

The reports have previously demonstrated that SDF-1α induced astrocytes and glioblastoma cell proliferation through the CXCR4 activation by ERK1/2 and Akt pathways [Ganju et al., 1998; Bonavia et al., 2003]. Recently, we also demonstrated that LRRC4 inhibited glioblastoma cell proliferation by reducing ERK/Akt/NF-κB signaling pathway [Wu et al., 2006a]. We further studied the role of LRRC4 on SDF-1α/CXCR4-induced ERK1/2 and Akt activation. We showed that in U251 cells transfected with mock or LRRC4, basal ERK1/2 activation was increased after SDF-1α stimulation (Fig. 3A), as observed in Akt activation

(Fig. 3B). However, re-expressed LRRC4 in U251 cells significantly suppressed SDF-1α/CXCR4-induced ERK1/2 and Akt activation (low panel in Fig. 3A,B). We also demonstrated that both PD98059 of a MEK inhibitor and LY294002 of a specific PI3K inhibitor, reduced both basal and SDF-1α-induced cell proliferation, chemotaxis, and invasion in U251 cells transfected with mock or LRRC4. LRRC4 synergized the inhibitory effect of both inhibitors, respectively (Fig. 3). In addition, PD98059 significantly reduced the basal [Wu et al., 2006a] and SDF-1α-induced ERK1/2 activation, LRRC4s synergy with PD98059 almost completely blocked the SDF-1α-induced ERK1/2 activation (Fig. 3A). At the same time, LY294002 significantly reduced the basal and SDF-1α-induced AKT activation; LRRC4s synergy with LY294002 almost completely blocked the SDF-1α-induced AKT activation (Fig. 3B). The data demonstrated that ERK1/2 and AKT were involved in the proliferative and invasive signal of SDF-1α/CXCR4, and LRRC4 significantly inhibited the SDF-1α/CXCR4-induced proliferation, chemotaxis, and invasion by reducing ERK1/2 and AKT signaling pathways.

Proteolytic enzymes such as MMPs are widely involved in matrix degradation in the context of motility and in vivo migration of normal and malignant cells [Bonavia et al., 2003]. High levels of the expression of MMPs have been reported in high-grade gliomas, where it is proposed that their action serves to facilitate invasion. Zhang et al. [2005b] reported that the SDF-1α promotes glioma invasiveness through MT2-MMP, but not the other MT-MMPs, MMP-2, or MMP-9. But in the current research, we showed that SDF-1α stimulation induced the activation of proMMP-2, and this activated effect was inhibited by LRRC4 (Fig. 4). Several reports indicate that the enhancement of human MMP-2 and MMP9 secretion involves the activation of ERK and Akt pathways [Kim et al., 2005; Jiang et al., 2006; Wang et al., 2006]. Moreover, LRRC4 synergized the inhibitory effect of the MEK inhibitor PD98059 or the PI3K inhibitor LY294002 after SDF-1α stimulation, suggesting that LRRC4 predominantly controls activation of proMMP-2 by SDF-1α/CXCR4-mediated ERK1/2 and Akt signaling pathway.

In conclusion, we demonstrate that CXCR4 is expressed in human glioblastoma U251 cell line, and that SDF-1α increases the proliferation, chemotaxis, and invasion in $CXCR4^+$ glioblastoma cells through the activation of ERK1/2 and Akt. Our results also indicate that reintroduction of LRRC4, as a glioma suppressive gene, in U251 cells (LRRC4 is absent in U251 cells [Wu et al., 2006a]) inhibits the expression of CXCR4 and SDF-1α/CXCR4 axis-mediated downstream intracellular pathways such as ERK1/2 and Akt leading to proliferative, chemotactic, and invasive effects. Furthermore, we provide evidence for proMMP-2 activation involvement in the SDF-1α/CXCR4 axis-mediated signaling pathway. LRRC4 evidently inhibits proMMP-2 activation by SDF-1α/CXCR4 axis-mediated ERK1/2 and Akt signaling pathway. Collectively, these results suggest a possible important "cross-talk" between LRRC4 and SDF-1α/CXCR4 axis-mediated intracellular pathways that can link signals of cell proliferation, chemotaxis, and invasion in glioblastoma, and may represent a new target for development of new therapeutic strategies in glioma.

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