LGR5/GPR49 is implicated in motor neuron specification in nervous system

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HIGHLIGHTS

- The biological role of LGR5 is closely related with neuron development and functions.
- LGR5 is specifically highly expressed in projection motor neurons in nervous system.
- Notch signal inhibited neurogenic potential and LGR5 expression in neural stem cells.
- Knockdown of LGR5 inhibited neurogenic potential of neural stem cells.
- LGR5 regulated the expression of key factors programing the identity of motor neurons.

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ABSTRACT

The biological roles of stem cell marker LGR5, the receptor for the Wnt-agonistic R-spondins, for nervous system are poorly known. Bioinformatics analysis in normal human brain tissues revealed that LGR5 is closely related with neuron development and functions. Interestingly, LGR5 and its ligands R-spondins (RSPO2 and RSPO3) are specifically highly expressed in projection motor neurons in the spinal cord, brain stem and cerebral. Inhibition of Notch activity in neural stem cells (NSCs) increased the percentage of neuronal cells and promoted LGR5 expression, while activation of Notch signal decreased neuronal cells and inhibited the LGR5 expression. Furthermore, knockdown of LGR5 inhibited the expression of neuronal markers MAP2, NeuN, GAP43, SYP and CHRM3, and also reduced the expression of genes that program the identity of motor neurons, including Isl1, Lhx3, PHOX2A, TBX20 and NEUROG2. Our data demonstrated that LGR5 is highly expressed in motor neurons in nervous system and is involved in their development by regulating transcription factors that program motor neuron identity.

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1. Introduction

LGR5 (leucine-rich repeat-containing G protein-coupled receptor 5 also known as GPR49) was recently identified as a stem cell marker for several tissues and cancers, including intestine and colon normal and cancer stem cells, hair follicle stem cells, etc. [1–4]. More recently, LGR5 was reported to be expressed in glioblastoma stem cells (GSCs), and contribute to the maintenance of GSCs [5,6]. However, the biological functions of LGR5 for stem cells are poorly acknowledged, even for the established stem cells that it marks, such as intestine and colon stem cells. The potential of LGR5 to identify stem cells in different kinds of tissues implies it may be a more universal stem cell marker, raising the interesting question that what is its biological function in nervous system?

By taking advantage of bioinformatics analysis, our previous study demonstrated that LGR5 is involved in the process of neurogenesis [6]. Biological experiments further confirmed that LGR5 regulate a cohort of genes involved in the network of neuronal and oligodendrocyte differentiation (NODRN) [6]. Interestingly, a positive feedback between LGR5 and OLIG2 was identified [6]. In addition, LGR5 is strongly regulated by neurogenin 1 [7], a proneural basic helix-loop-helix (bHLH) transcription factor that is important for neural stem cell (NSC) differentiation. All of these results imply that LGR5 may play a role in the neuronal differentiation and maturation. Here, we further studied the biological role of LGR5 in nervous system, and revealed that LGR5 is involved in the process of neuronal differentiation and maturation, especially in the development of motor neurons in central nervous system (CNS).

2. Methods and materials

2.1. ARACNe network reconstruction and bioinformatics analysis

ARACNe (Algorithm for the Reconstruction of Accurate Cellular Networks), an information-theoretic algorithm for inferring transcriptional interactions [8], was used to identify a repertoire of candidate transcriptional regulators of interesting genes, as described previously [9]. Expression profiles of normal brain tissue used in the analysis were from GSE15745 (Gene expression profiles derived from frozen tissue samples of the cerebellum (CRBLM), frontal cortex (FCTX), caudal pons (PONS) and temporal cortex (TCTX) obtained from 150 neurologically normal subjects) [10] and GSE11882 (Gene expression profiles derived from frozen tissue samples in the hippocampus (HC), entorhinal cortex (EC), superior frontal gyrus (SG), and postcentral gyrus (PCG) across the lifespan of 63 cognitively intact individuals from 20–99 years old) [11]. First, candidate interactions between a transcription factor (TF, x) and its potential target (y) are identified by computing pairwise mutual information, MI[x; y], using a Gaussian kernel estimator and by thresholding the MI based on the null-hypothesis of statistical independence ($p < 0.05$, Bonferroni corrected for the number of tested pairs). Then, indirect interactions are removed using the data processing inequality (DPI) analysis with a tolerance of 20%, a well-known property of the mutual information.

2.2. Culture of primary neural stem cells (NSCs)

Culturing of human fetal cortical NSCs were isolated from two spontaneous aborted fetuses (8–12 weeks) as described previously [9,12]. The study protocol was approved by Institutional Review Committee of Xijing Hospital of the Fourth Military Medical University. Written informed consent was obtained from patients. Briefly, samples were dissociated to a single cell suspension using a fire-polished Pasteur pipette and cultured in serum free medium (SFM) consisting of DMEM-F12 medium, EGF (20 ng/mL; Invitrogen, Carlsbad, CA), bFGF (20 ng/mL; Invitrogen) and B27 (1:50; Invitrogen).

2.3. Inhibition and activation of Notch signal

The highly active γ-secretase inhibitor, N-[N-(3, 5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), purchased from Sigma–Aldrich Corporation (USA), was used to inhibit the Notch signal activity.

To activate Notch signal, a truncated, constitutively active form of the NOTCH2 receptor (NICD2) was subcloned to adenovius. Breifly, cytoplasmic portion of the human Notch2 receptor (amino acids 1747–2531) was inserted into a mammalian expression vector pcDNA3.1 (Invitrogen) to create a constitutively active form

of Notch2 (pcDNA3.1-NICD2). Then the plasmid pcDNA3.1-NICD2 was transfected into the cells with Lipofectamine™ 2000 according to the manufacturer's instructions. Vector pcDNA3.1 was used as control.

2.4. siRNA transfection

LGR5 knockdown by siRNA was performed as described previously [6]. Briefly, the NSC spheres were dissociated into single cells and plated on poly-L-ornithine (50 mg/ml, P3655, Sigma Chemical) coated wells (50,000 cells per 24–well platewell) to attach for 24 h. Then the cells were transfected by Stealth small interfering RNAs (siRNA) targeting LGR5 or non-targeting RNA sequence (Invitrogen Lifetechnologies, USA) with Oligofectamine™ RNAiMAX Reagent according to the manufacturers protocol (Invitrogen). After 24 h of incubation, the cells were washed and detached from the wells and placed back to SFM medium. The efficiency of siRNA interference was evaluated by qPCR after 72 h of transfection.

2.5. Immunofluorescence

Immunofluorescence was performed on cultured and differentiated NSCs as described previously [12]. Briefly, undifferentiated neural spheres were plated onto poly-L-lysine coated glass coverslips for 4 h, fixed with 4% paraformaldehyde, and incubated with NeuN (1:1000, mouse monoclonal IgG1; Chemicon, Temecula, CA), GFAP (1:5000, rabbit polyclonal; Abcam), and MAP2 (1:1000, Santa Cruz) antibody overnight at 4 °C. Texas Red goat anti-rabbit (Molecular Probes, Invitrogen, Carlsbad, CA) was used as secondary antibody. Counterstaining with Hoeschst 33342 (Sigma, St. Louis, MO) was additionally performed to permit counting of cell nuclei.

2.6. qPCR

qPCR was performed as described previously [9,13]. briefly, RNA was extracted from cultured cells using Trizol Reagent (Invitrogen), and reverse transcribed into cDNA. qPCR analysis was performed on an ABI7700 using SYBR Green PCR Core Reagents in 20 μL volume (Applied Biosystems, Warrington, UK). Primer sequences for qPCR studies are shown in Supplementary Table 1. All samples, including template-free controls, were assayed in triplicate and the relative amount of target transcripts normalized to the number of human β-actin transcripts found in the same sample. Specificity was verified by melting curve analysis and agarose gel electrophoresis. Relative fold changes were calculated using the $\Delta\Delta C_t$ method by using the threshold cycle values of each sample.

2.7. Western blotting

Cultured cells were lysed in SDS sample buffer, and 20 μg proteins were run on 6% SDS-PAGE gel and transferred to a nitrocellulose membrane. Blots were blocked in PBS containing 5% nonfat dry milk powder and incubated overnight at 4 °C with primary antibody of LGR5 (1:1000, Abcam) or GAPDH (1:500,000; Abcam). Blots were then washed with PBS containing 0.1% Tween 20 (PBST) and incubated in secondary antibodies coupled to peroxidase. After washing in PBST, blots were developed with chemiluminescence according to the manufacturer's instructions (enhanced chemiluminescence, Amersham Biosciences, GE Healthcare, France). All Western blot analyses were done in duplicates.

2.8. Statistical analysis

Statistical analyses were performed using Student's t-tests and one-way analysis of variance with least squared difference post hoc tests, as appropriate. All P values are two-tailed. A value of