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SOX9-mediated upregulation of LGR5 is important for glioblastoma tumorigenicity

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ABSTRACT

LGR5 plays an important role in the self-renewal of stem cells and is used as a marker identifying self-renewing stem cells in small intestine and hair follicles. Moreover, LGR5 has been reported to be overexpressed in several cancers. SOX9 is a transcription factor that plays a key role in development, differentiation and lineage commitment in various tissues. It has also been reported that SOX9 is overexpressed in a variety of cancers and contributes to their malignant phenotype. Here we show that LGR5 is required for the tumorigenicity of glioblastoma cells. We further show that SOX9 is upregulated in glioblastoma cells and directly enhances the expression of LGR5. We also demonstrate that knockdown of SOX9 suppresses the proliferation and tumorigenicity of glioblastoma cells. These results suggest that SOX9-mediated transcriptional regulation of LGR5 is critical for the tumorigenicity of glioblastoma cells. We speculate that the SOX9-LGR5 pathway could be a potentially promising target for the therapy of glioblastoma.

1. Introduction

Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), also known as GPR49, contains a large extracellular domain and a seven-transmembrane domain, characteristic of G protein-coupled receptors [1]. R-spondins (RSPOs) have recently been identified as ligands for LGR4/5/6 and both RSPO-LGR complexes and Wnt ligands can activate Wnt signaling by binding to Frizzled-LRP receptor complexes at the cell membrane [2–4]. Furthermore, LGR5 is required for the self-renewal of stem cells and is used as a marker for self-renewing stem cells in small intestine and hair follicles [5,6]. In addition, it has been reported that LGR5 is overexpressed in several cancers, including colorectal tumors and glioblastomas [7–9]. It has also been reported that LGR5 is required for the survival of glioblastoma cells and its expression levels are correlated with poor prognosis in glioblastoma patients [7,10].

Sry-related high-mobility group (HMG) box 9 (SOX9) is a transcription factor that plays key roles in development, differentiation and lineage commitment in various tissues, including the intestine, liver and pancreas [11–13]. For example, Sox9 plays essential roles in the proliferation of stem/progenitor cells and in the differentiation of Paneth cells in the intestinal epithelium [12]. Furthermore, it has been reported that SOX9 is overexpressed in a variety of cancers and contributes to their malignant phenotype [14,15]. It has also been reported that SOX9 is required for the proliferation of malignant glioma and that overexpression of SOX9 is closely associated with poor prognosis [15].

In this study, we investigated the mechanisms underlying the increased expression of LGR5 in glioblastoma cells. We find that SOX9 directly upregulates the transcription of LGR5, and our results suggest that this upregulation is important for the proliferation and tumorigenicity of glioblastoma.

2. Materials and methods

2.1. Ethical statement

The protocols used in this study were approved by the Ethics Committee of Tokyo University Hospital and the Institute of Molecular and Cellular Biosciences. Written informed consent was obtained from all individuals who participated in the study. Mouse experiments conformed to the Guide for the Care and Use of

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Laboratory Animals published by the US National Institutes of Health and was approved by the Ethics Committee of Institute of Molecular and Cellular Biosciences, The University of Tokyo. All experiments were designed to minimize the number of animals used and their discomfort.

2.2. Cell culture

Glioblastoma samples were obtained from patients who had provided written informed consent and who had undergone surgical treatment at the University of Tokyo Hospital. GB2 and GB11 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Life Technologies) containing B27 supplement minus vitamin A (Life Technologies), and also containing epidermal growth factor, and fibroblast growth factor 2 (20 ng/ml each; Wako Pure Chemicals Industries) [16].

2.3. MTT assay

MTT assays were performed according to the manufacturer's instruction (Roche). Briefly, GB2 and GB11 cells infected with an shRNA-expressing lentivirus were cultured for 6 days, trypsinized and plated in 24-well tissue culture plates. After culturing for 120 h, MTT (Roche) was added directly to each well at a final concentration of 0.5 mg/ml. Following the addition of MTT, cells were incubated for 4 h at 37 °C. The supernatants were removed from the wells and formazan crystal was dissolved in 500 μl of 10% sodium dodecyl sulfate in 0.01 M HCl. The absorbance of each well was measured at 570 nm using a luminometer (Mithoras LB 940, BERTHOLD).

2.4. RNAi

For lentivirus production, the lentiviral vector CS-Rfa-CG, harboring an shRNA driven by the H1 promoter, was transfected with the packaging vectors pCAG-HIV-gp and pCMV-VSV-G-RSV-Rev into HEK293FT cells using Lipofectamine 2000 Transfection Reagent (Life Technologies). All plasmids were kindly provided by H. Miyoshi (RIKEN BioResource Center). Virus supernatants were purified by ultracentrifugation at 25,000 rpm at 4 °C for 90 min (SW28 rotor; Beckman Coulter Genomics). The shRNA target sequences were as follows: SOX9#5, 5′- GCAAGCTCTGGAGACTTCTGA -3′; SOX9#6, 5′- GCGACGTCATCTCCAACATCG -3′; LGR5#1, 5′- ATGGACGACCTTCATAAGAAAGA -3′. The infection efficiency of the lentiviruses was more than 95%, as judged by GFP fluorescence. Cells were transfected with the siRNA Library (Life Technologies) using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions. 15 nM siRNA was added to each well.

2.5. Real-time PCR

Total RNA was extracted using TRIsure (BIOLINE) and 500 ng was used to synthesize cDNA using the PrimeScript RT Master Mix (TaKaRa). Real-time PCR was performed in duplicate using the Light Cycler 480 System (Roche) and Light Cycler480 SYBER Green I Master (Roche). The results were normalized to hypoxanthine phosphoribosyltransferase 1 (HPRT1) or TATA box binding protein (TBP) expression and expressed as ΔΔCt. Primer sequences are listed in Supplementary Table S1.

2.6. Sphere formation assay

GB2 and GB11 cells were infected with shRNA-expressing lentiviruses, cultured for 3 days, re-plated in 96-well tissue culture plates (1,000, 500 or 200 cells per well) and then cultured for 2 weeks. Spheres were photographed by an In Cell Analyzer 2000 system (GE Healthcare) and analyzed using Developer 1.9.1 software (GE Healthcare) [17].

2.7. Intracranial xenografts

One week after lentivirus infection, RNA was extracted from a portion of the infected cells and the knockdown efficiency was quantified by qRT-PCR. The knockdown cells (1.0×10^4 cells) were injected stereotactically into the right frontal lobes of 5-week-old nude mice under general anesthesia (BALB/cAJclnu/nu, CLEA Japan Inc., Tokyo, Japan) ($n = 4$). The injection coordinates were 2 mm to the right of the midline, 1 mm anterior to the coronal suture and 3 mm deep. Mice were monitored for up to 6 months. All animal experimental protocols were performed in accordance with the policies of the Animal Ethics Committee of the University of Tokyo.

2.8. Plasmid construction

The SOX9 cDNA was purchased from ATCC. The reporter constructs were generated as previously described [16]. Briefly, the promoter regions of *LGR5* were amplified by PCR using the corresponding specific primers and cloned into the pGL3-basic vector (Promega). The mutant construct LGR5-P3-mut, which contains a mutated SOX9-binding site in the *LGR5* promoter, was generated by site-directed mutagenesis. All PCR products were amplified using KOD-Plus-Neo (TOYOBO).

2.9. Luciferase assay

Luciferase assays were performed as previously described [18]. Briefly, GB2 cells were transfected with a luciferase-reporter plasmid and cultured for 24 h. Cells were lysed and firefly luciferase activity was measured with the Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

2.10. Antibodies

Rabbit polyclonal antibody to SOX9 was obtained from Millipore (AB5535: Bedford, MA, USA). Rabbit polyclonal antibody to GFP was from Santa Cruz Biotechnology (sc-8334: Santa Cruz, CA, USA). Purified Rabbit IgG was from Millipore (PP64: Bedford, MA, USA).

2.11. Chromatin immunoprecipitation assay

ChIP assays were performed as described previously [16]. Briefly, DNA fragments immunoprecipitated with anti-SOX9 antibody or rabbit IgG (2 μg) were analyzed by real-time PCR using primers directed against a region containing the predicted SOX9-binding site in the *LGR5* promoter region. A region in the promoter region of *Actin* was used as a negative control. Primer sequences are listed in Supplementary Table S1.

2.12. Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue blocks were sectioned (6 μm thick) onto slides and then deparaffinized. Slides were microwaved for 15 min in target retrieval solution (pH 6.0; Dako). Internal peroxidases were blocked by incubation in 0.3% H_2O_2 solution in methanol for 20 min. Non-specific staining was blocked by a 30 min incubation with blocking solution (10% Goat serum, 0.1% Tween20 in PBS). Sections were immunostained with the ABC kit (VECTOR). Anti-GFP antibodies were used at a dilution of 1:200. Sections were exposed to diaminobenzidine peroxidase substrate (Funakoshi) and counterstained with Mayer's