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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

hematoxylin. All images were obtained by bright field microscopy AX80 (Olympus).

2.13. Kaplan–Meier analysis

Survival of mice was evaluated by Kaplan–Meier analysis. *P* values were calculated using a log rank test.

2.14. Statistical analysis

The data presented in bar graphs are the means ± s.d. of three independent experiments or multi wells. Statistical analyses were performed using the Student's t-test.

3. Results and discussion

3.1. Knockdown of LGR5 suppresses glioblastoma cell growth, sphere formation and tumorigenicity

We previously isolated glioblastoma cells from patients and passaged them under serum-free conditions to obtain two glioblastoma cell lines, GB2 and GB11 [16,17]. These cell lines were highly tumorigenic when transplanted into nude mice and their tumorigenicity was retained even after passage under adherent culture conditions on laminin-coated dishes [17].

Previous studies have reported that LGR5 is highly expressed in glioblastomas and is required for their proliferation [7]. To examine whether LGR5 is required for the tumor-initiating property of glioblastoma cells, we infected GB2 cells with a lentivirus expressing a small hairpin RNA (shRNA) targeting LGR5. We found that knockdown of LGR5 resulted in a decrease in both proliferation and sphere formation (Fig. 1A, B and C). When we transplanted GB2 cells stably expressing the LGR5-knockdown shRNA into the frontal lobes of nude mice, we found that these mice survived longer than mice transplanted with control parental GB2 cells (Fig. 1D).

3.2. SOX9 upregulates LGR5 expression in glioblastoma cells

To clarify the mechanisms underlying the upregulation of LGR5 in glioblastoma, we generated luciferase reporter constructs in which short fragments of the LGR5 promoter region (LGR5-P1, -P3 and -P4) were inserted upstream of the luciferase gene (Fig. 2A). When transfected into GB2 cells, LGR5-P1 and -P3 exhibited higher activity than LGR5-P4 or the control vector (pGL3-basic) (Fig. 2B). This result suggests that the region between −300 and the transcription start site (TSS) possesses promoter activity.

To identify transcription factors involved in the upregulation of LGR5 expression in glioblastoma, we first searched for transcription factor-binding motifs in the −300 ~ TSS region of the LGR5 promoter. We found potential binding sites for 40 candidate transcription factors, and from these we selected 21 that were highly expressed in GB2 cells. We next asked whether silencing of any of these by small interfering RNA (siRNA) would cause a reduction in LGR5 expression and found that knockdown of the transcription factor SOX9 caused the most significant reduction in LGR5 transcription (Fig. 2C). We therefore confirmed that knockdown of SOX9 reduced the amount of LGR5 expression by another siRNA (Fig. 2D).

To confirm that SOX9 transactivates LGR5, we generated a mutant promoter construct in which the predicted SOX9 binding site was abolished (LGR5-P3-mut) and examined the effects of SOX9 knockdown on the activities of luciferase reporters (LGR5-P3 and P3-mut) (Fig. 3A and B). Silencing of SOX9 in GB2 cells downregulated the activity of the LGR5-P3. Furthermore, mutation of the SOX9 binding site within LGR5-P3 (LGR5-P3-mut) resulted in reduced promoter activity and abolished the inhibitory effect of SOX9 knockdown on the activity of luciferase reporter. To investigate whether SOX9 regulates LGR5 transcription directly, we performed chromatin immunoprecipitation (ChIP) assays on GB2 cells using anti-SOX9 antibody. We detected SOX9 binding to a DNA fragment containing the LGR5 promoter SOX9-binding site (Fig. 3C).

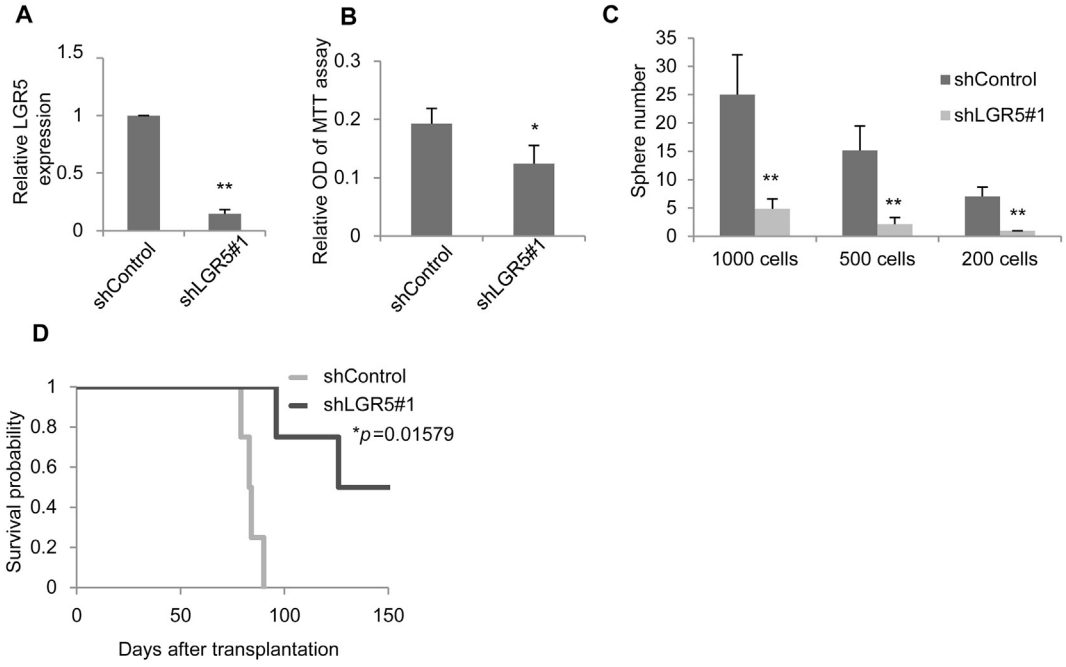

Fig. 1. LGR5 is required for the tumorigenicity of glioblastoma cells. (A) qRT-PCR analysis of LGR5 expression in GB2 cells infected with a lentivirus expressing an shRNA targeting LGR5. HPRT1 was used as an internal control. Results are expressed as the means ± s.d. of three independent experiments. $^{**}p < 0.01$, Student's t-test. (B) Growth of GB2 cells infected with a lentivirus expressing an shRNA targeting LGR5. Growth was quantified by MTT assay. Results are shown as average of 3 wells ±s.d. $^{*}p < 0.05$, Student's t-test. (C) The numbers of spheres formed by GB2 cells infected with a lentivirus expressing an shRNA targeting LGR5. Results are shown as an average of 8 wells ±s.d. $^{**}p < 0.01$, Student's t-test. (D) Kaplan–Meier survival curves of mice transplanted with 1.0×10^4 GB2 cells infected with a lentivirus expressing an shRNA targeting LGR5 or control shRNA (n = 4).

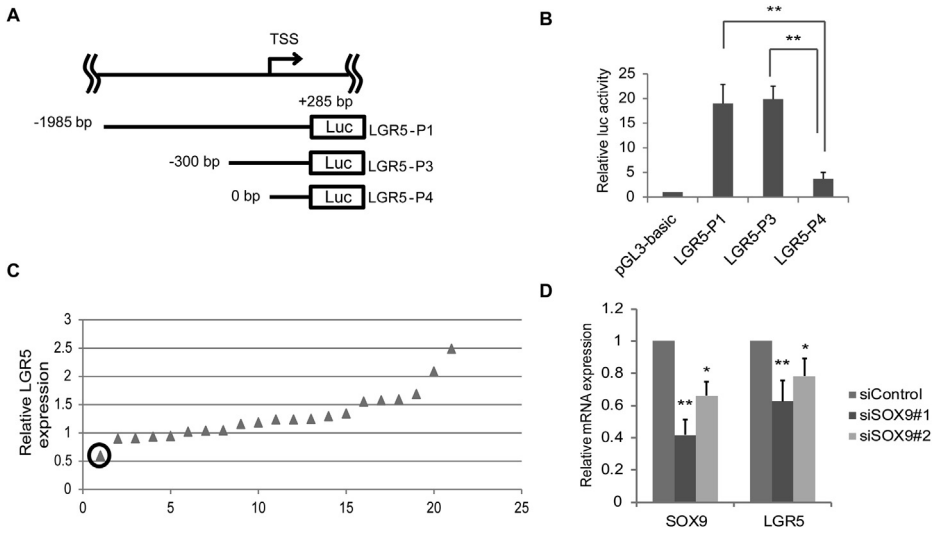

Fig. 2. siRNA screen for transcription factors involved in the regulation of LGR5 expression. (A) Schematic diagram of the *LGR5* promoter region and reporter constructs used for luciferase assays. Fragments of the *LGR5* promoter were cloned upstream of the luciferase (Luc) gene. (B) GB2 cells were transfected with reporter constructs containing *LGR5* promoter sequences and subjected to luciferase assays. pRL-SV40 vector was used as an internal control. Results are expressed as the means ± s.d. of three independent experiments. $^{**}p < 0.01$, Student's t-test. (C) GB2 cells were transfected with siRNAs targeting 21 transcription factors and LGR5 mRNA expression levels were assessed by qRT-PCR. HPRT1 was used as an internal control. The black circle indicates the data obtained from cells transfected with an siRNA targeting SOX9. (D) qRT-PCR analysis of SOX9 and LGR5 expression in GB2 cells transfected with siRNAs targeting SOX9. HPRT1 was used as an internal control. Results are expressed as the means ± s.d. of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, Student's t-test.

3.3. Knockdown of SOX9 reduces the proliferation, sphere formation and tumorigenicity of glioblastoma cells

It has recently been shown that SOX9 is required for the proliferation of glioma cells and that its expression levels are correlated with poor prognosis [15]. We therefore investigated the role of SOX9 on GB2 cell growth and sphere formation. We found that suppression of SOX9 expression by shRNA resulted in decreased LGR5 expression, cell proliferation and sphere-forming capacity (Fig. 4A, B and C). Similar results were obtained with GB11 cells (Supplementary Fig. S1). We next examined whether SOX9 is important for the tumorigenicity of GB2 cells. Mice were intracranially transplanted with GB2 cells that had been infected with a lentivirus expressing an shRNA targeting SOX9. These mice were found to survive longer than those transplanted with the parental GB2 cells (Fig. 4D). Histological analysis revealed that the SOX9-knockdown GB2 cells formed smaller tumors than the control parental GB2 cells (Fig. 4E). These results suggest that SOX9 plays a key role in the tumorigenicity of glioblastoma cells.

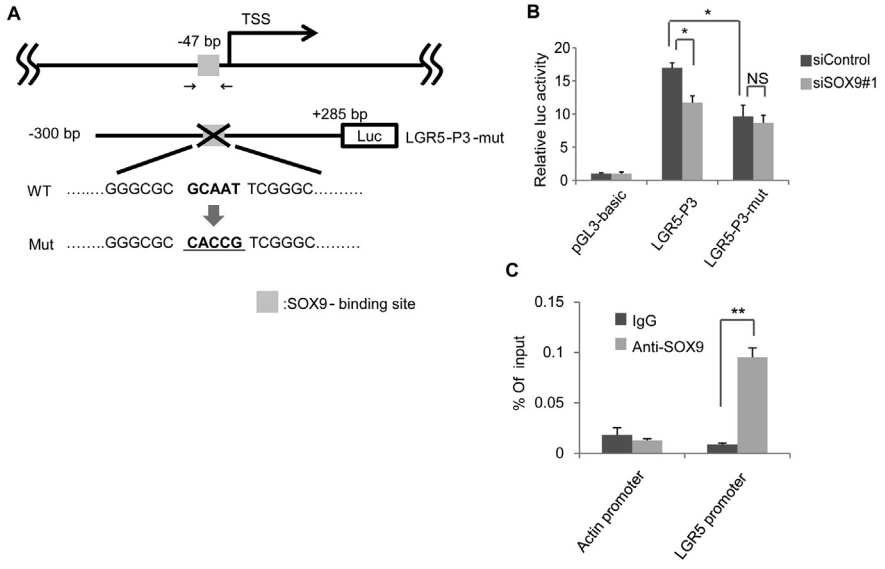

Fig. 3. SOX9 upregulates LGR5 expression in glioblastoma cells. (A) Schematic illustration of the *LGR5* promoter region and reporter constructs. Mutations were introduced into the potential SOX9-binding site as shown by bold letters. The potential SOX9-binding site is indicated by the gray box. The positions of the primers used for ChIP assays are indicated by arrows. The primer sequences are provided in Supplementary Table S1. (B) GB2 cells were transfected with siRNA targeting SOX9 along with wild-type or mutated *LGR5* reporter construct (LGR5-P3 and P3-mut), and were subjected to luciferase assays. pRL-SV40 vector was used as an internal control. Results are expressed as the means ± s.d. of three independent experiments. $^{*}p < 0.05$; NS, not significant, Student's t-test. (C) ChIP assays were performed on GB2 cells using anti-SOX9 antibody or control IgG. The promoter region of *Actin* was used as a negative control. Results are expressed as the means of the percentage of the input ±s.d. of three independent experiments. $^{**}p < 0.01$, Student's t-test.

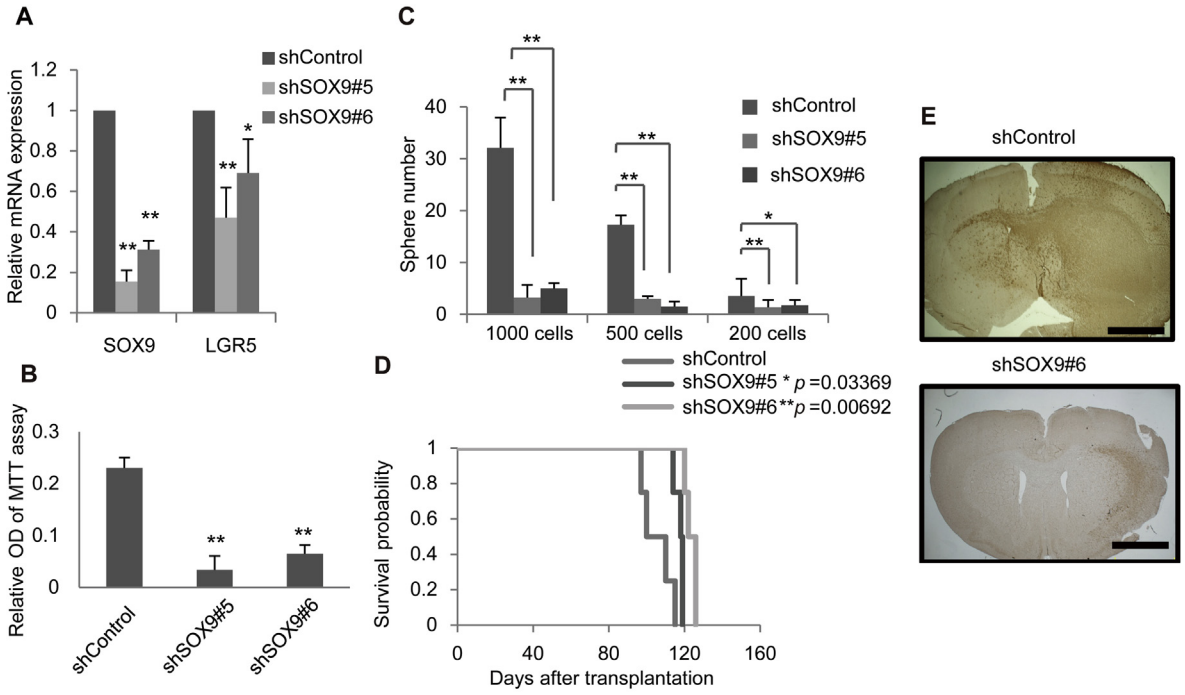

Fig. 4. SOX9 is required for the tumorigenicity of glioblastoma cells. (A) qRT-PCR analysis of SOX9 and LGR5 expression in GB2 cells infected with a lentivirus expressing an shRNA targeting SOX9. TBP was used as an internal control. Results are expressed as the means ± s.d. of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, Student's t-test. (B) Growth of GB2 cells infected with a lentivirus expressing an shRNA targeting SOX9. Growth was quantified by MTT assay. Results are expressed as the means ± s.d. of three independent experiments. $^{**}p < 0.01$, Student's t-test. (C) The numbers of spheres formed by GB2 cells infected with an shRNA targeting SOX9. Results are shown as an average of 8 wells ±s.d. $^{*}p < 0.05$, $^{**}p < 0.01$, Student's t-test. (D) Kaplan–Meier survival curves of mice transplanted with 1.0×10^4 GB2 cells infected with a lentivirus expressing an shRNA targeting SOX9 (n = 4). (E) Histological analysis of tumors developed in the mice in (D). Tissue sections were stained with anti-GFP antibody. The scale bars represent 2 mm.

We have previously reported that LGR5 transcription is directly activated by the transcription factor GATA6 in colorectal cancer cells and that the GATA6-LGR5 pathway is critical for colorectal tumorigenesis [19]. However, GATA6 is expressed at very low levels in glioblastoma [20], whereas SOX9 expression is elevated in glioblastoma compared to non-neoplastic tissues [15]. We have shown in this study that SOX9 directly activates the expression of LGR5 in glioblastoma and that the SOX9-LGR5 pathway is important for the sphere-forming capacity and tumorigenicity of glioblastoma cells. Since both SOX9 and LGR5 have been shown to play important roles in the self-renewal of stem/progenitor cells, we speculate that the SOX9-LGR5 pathway is important for the stem-like properties of glioblastoma cells. If so, this would make the SOX9-LGR5 pathway a potentially promising target for the therapy of glioblastoma.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.012.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.012.

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