Effects of a monoclonal antibody against (pro)renin receptor on gliomagenesis

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Abstract

Glioblastoma is characterized by a strong self-renewal potential and poor differentiated state. We have reported previously that the (pro)renin receptor [(P)RR] is a potential target for glioma therapy by silencing the (P)RR gene. Here, we have examined the effects of a monoclonal antibody against (P)RR on gliomagenesis.

Methods: Human glioma cell lines (U251MG and U87MG) and a glioma stem cell line (MGG23) were used for the *in vitro* study. The expressions of the Wnt/β-catenin signaling pathway (Wnt signaling pathway) components and stemness markers were measured by Western blotting. The effects of the (P)RR antibody on cell proliferation, sphere formation, apoptosis and invasion were also examined. Subcutaneous xenografts were also examined in nude mice.

Results: Treatment with the (P)RR antibody reduced expression of Wnt signaling pathway components and stemness markers. Furthermore, the (P)RR antibody reduced cell proliferation and decreased sphere formation significantly. The treatment also suppressed invasion and induced apoptosis. In a subcutaneous xenograft model, systemic administration of the (P)RR antibody reduced tumor volume significantly.

Conclusion: These data show that treatment with the (P)RR antibody is a potential therapeutic strategy for treating glioblastoma.

Introduction

Glioblastoma is an aggressive malignant human brain tumor [1]. The prognosis for patients with glioblastoma is unfavorable. Despite modern treatment protocols such as surgical resection or a combination of radiation therapy and temozolomide chemotherapy, the median survival time is 15 months with only 27% of patients living longer than two years following diagnosis [2, 3]. Previous studies have shown that glioblastoma is caused by a small number of specific cancer stem cells (CSCs) that form a tumor, which have potential for high tumorgenicity, self-renewal and multi-differentiation [4–6]. According to the CSCs hypothesis, eliminating glioma stem cells (GSCs) is an attractive therapy for treating glioblastoma [6–8].

The Wnt/β-catenin signaling pathway (Wnt signaling pathway) mainly contributes to GSC stemness, proliferation and survival. Since the Wnt signaling pathway is strongly involved in pivotal biological characters of glioblastoma, suppression of the Wnt signaling pathway offers a unique opportunity for attenuating gliomagenesis [9, 10, 11].

The (pro)renin receptor [(P)RR] was discovered and cloned by Nguyen et al. [12]. Previous studies have revealed that (P)RR is an integral component of the Wnt receptor complex and (P)RR binds to Frizzled and LDL receptor- related protein 6 (LRP6) in the Wnt receptor complex involved in the development of the central nervous system [13, 14, 15]. Furthermore, Hirose et al. showed that neuron-specific (P)RR

knockout mice inhibit stem cell self-renewal [15]. (P)RR is also aberrantly expressed in several cancers including glioblastoma [16–18]. We have shown previously that aberrant (P)RR expression activates the Wnt signaling pathway, which correlates with the malignancy of glioma. *In vitro* studies have also shown that (P)RR silencing with siRNA reduces the proliferative capacity in several human glioma cells [16].

More recently, we have developed a monoclonal antibody against the extracellular domain of (P)RR, which interacts with LRP6 of Wnt components [19]. In human pancreatic ductal adenocarcinoma [19] and colorectal cells [18], the (P)RR antibody [(P)RR Ab] remarkably suppressed tumorigenesis by inhibiting activation of the Wnt signaling pathway. In the present study, both *in vitro* and *in vivo* studies were conducted to examine the effect of this monoclonal antibody against (P)RR on gliomagenesis. In particular, we have focused on the specific role of (P)RR in the maintenance of glioma stem cells.

Results

Positive correlation between SOX2 and (P)RR expression in glioma

Immunohistochemistry revealed cytoplasmic (P)RR expression and nuclear SOX2 expression in human glioma tissues (**Figure 1A**). Supplementary Table S1 summarizes the detailed characteristics, PS and IS of the 56 glioma patients. (P)RR expression increased significantly along with the WHO grade [the mean PS was 9.3 ± 9.5% for grade , 41.5 ± 23.4% for grade ($P < 0.01$) and 64.6 ± 25.5% ($P < 0.01$) for grade , and the mean IS was 0.9 ± 0.6 for grade , 1.6 ± 0.8 for grade ($P = 0.03$) and 1.5 ± 0.8 for grade ($P = 0.03$), **Supplementary Fig S1A**]. SOX2 expression tended to increase along with the WHO grade [the mean PS was 11.3 ± 9.5% for grade , 24.2 ± 24.9% for grade ($P = 0.11$) and 24.5 ± 25% for grade ($P = 0.02$), and the mean IS was 1.1 ± 0.6 for grade , 1.4 ± 0.9 for grade ($P = 0.44$) and 1.6 ± 0.8 for grade ($P = 0.09$), **Figure 1B, C**]. SOX2 expression positively correlated with (P)RR expression (Spearman's correlation coefficient, $r = 0.40$, $P < 0.01$, **Figure 1D**). PS of (P)RR and SOX2 expression increased with tumor malignancy irrespective of isocitrate dehydrogenase (*IDH)*1^{R132H} or 1p19q status (**Supplementary Fig. S2**).

Monoclonal (P)RR Ab downregulates the components of the Wnt signaling pathway and stemness markers

The Wnt/β-catenin signaling pathway is a key regulator of cancer cells [24]. Genes such as cyclin D1 and c-myc are activated following the molecular interaction between β-catenin and lymphoid enhancer binding factor (LEF)/T-cell factor in the nucleus (TCF) [25]. We have already demonstrated that (P)RR is an important component of the canonical Wnt signaling pathway in human glioblastoma cells by using (P)RR siRNA [16]. Therefore, we investigated the effect of a monoclonal antibody against (P)RR on the expression of active (nonphosphorylated) β-catenin and c-myc located downstream of the Wnt signaling pathway. A decrease in (P)RR expression caused consistent downregulation of β-catenin and c-myc expression in human glioblastoma cell lines U251MG and U87MG and the human GSC cell line MGG23 (**Figure 2**). C-myc, Oct3/4 and SOX2 are pivotal transcriptional factors responsible for stem cell

maintenance [26]. Expression of these proteins was also downregulated in U251MG, U87MG and MGG23 cells following treatment with the (P)RR Ab (**Figure 2**).

(P)RR Ab suppresses cell viability in human glioblastoma cell lines

Cell proliferation was evaluated by the WST-1 assay in U251MG and U87MG cells. This assay revealed that treatment with the (P)RR Ab dose-dependently inhibited the cell viability of U251MG cells [the mean relative cell viability was 1 ± 0.06 for the control, 0.88 ± 0.03 for a (P)RR Ab treatment of 100 μg/mL (P < 0.01), 0.81 ± 0.04 for 200 μg/mL (P = 0.01) and 0.76 ± 0.02 for 400 μg/mL (P < 0.001), n = 4, **Figure 3A**] and U87MG cells [the mean relative cell viability was 1 ± 0.04 for the control, 0.86 ± 0.05 for a (P)RR Ab treatment of 100 μg/mL (P = 0.04), 0.78 ± 0.04 for 200 μg/mL (P = 0.03) and 0.73 ± 0.03 for 400 μg/mL (P = 0.02), n = 4, **Figure 3B**]. We also investigated proliferation by direct cell counting in human glioblastoma cell lines. The (P)RR Ab decreased significantly the number of viable U251MG cells [the mean cell number was 2.19 ± 0.17 × 10^4 for the control, 1.24 ± 0.32 × 10^4 for a (P)RR Ab treatment of 200 μg/mL (P = 0.02) and 0.88 ± 0.13 for 400 μg/mL (P = 0.01), n = 3, **Figure 3A**] and U87MG cells [the mean cell number was 8.59 ± 0.55 × 10^4 for the control, 4.19 ± 0.63 × 10^4 for a (P)RR Ab treatment of 200 μg/mL (P = 0.02) and 2.85 ± 0.38 for 400 μg/mL (P = 0.004), n = 3, **Figure 3B**]. The (P)RR Ab also affected the cell morphology of human glioblastoma cell lines. U251MG cells treated with the (P)RR Ab had longer dendrites when compared with that of control cells (**Figure 3A**). U87MG cells treated with the (P)RR Ab changed cell shape with more edematous than that observed for control cells (**Figure 3B**). These findings demonstrated that the (P)RR Ab clearly suppressed human glioblastoma cell viability.

Antitumor effects of the (P)RR Ab on engrafted U87MG cells

To examine the effects of the (P)RR Ab on glioblastoma tumor growth *in vivo*, we subcutaneously injected U87MG cells into the right flank of immunodeficient mice and treated the mice with either the (P)RR Ab or supernatant control medium. Twenty days after injection, the mean tumor volume for mice treated with the (P)RR Ab was significantly smaller than the tumors present in the control mice (1730 ± 399 mL *vs.* 2739 ± 707 mL, P < 0.01, n = 7, **Figure 3C**). The body weight of mice was very similar between groups (24.1 ± 1.1 g *vs.* 25.3 ± 1.1 g, P = 0.07, n = 7**, Supplementary Fig. S3A**). Resected tumors treated with control medium had more irregular shapes and a larger necrotic condition than tumors treated with the (P)RR Ab (**Figure 3C**). Although there was no significant difference in the mean tumor weight because necrotic tumors were lost when we resected control tumors, the weights of tumors treated with the (P)RR Ab were slightly reduced (2.25 ± 0.47 g *vs.* 2.17 ± 1.05 g, P = 0.86, n = 7, **Supplementary Fig. S3C**). These results demonstrated that the (P)RR Ab attenuated the growth of tumors in glioblastoma significantly.

(P)RR Ab inhibits human glioma stem cell features

We performed the sphere formation assay to confirm the effect of the (P)RR Ab. Data showed that the sphere size decreased significantly [the mean relative sphere size was 1 ± 0.09 for the control, 0.72 ± 0.09 for a (P)RR Ab treatment of 80 μg/mL (P = 0.01) and 0.47 ± 0.04 for 160 μg/mL (P = 0.002), n = 4, **Figure 4A**], and the quantity of spheres increased significantly in MGG23 cells treated with the (P)RR Ab [the

mean sphere quantity was 26.8 ± 8.2 for the control, 39 ± 12.2 for a (P)RR Ab treatment of 80 μg/mL (P = 0.08) and 83.8 ± 9.8 for 160 μg/mL (P = 0.007), n = 4, **Figure 4A**]. Cell proliferation was evaluated by the MTT assay using MGG23 cells. The (P)RR Ab inhibited cell proliferation in a time-dependent manner [the mean cell viability was 0.56 ± 0.04 *vs*. 0.33 ± 0.02, P = 0.003 (day 2), and 0.76 ± 0.02 *vs*. 0.55 ± 0.03, P < 0.001 (day 4), n = 4, **Figure 4B**]. Thus, these data implies that the cell viability of smaller spheres decreases under (P)RR Ab treatment. Previous studies have indicated that stem cells give rise to larger spheres while progenitor cells lost the ability of self-renewal have a character of smaller spheres [27], which implies that the sphere size is more important biological feature of stem cells compared to the sphere number. These data have suggested that the size of the spheres plays a more critical role in the proliferation of sphere forming cells [28]. Previous studies support our data. These data indicated that the (P)RR Ab suppressed a large sphere formation and viability of glioblastoma stem cells significantly.

(P)RR Ab induces apoptosis

We performed flow cytometry and caspase-3 assays to evaluate apoptosis in U251MG, U87MG and MGG23 cells. FITC-labeled annexin was used to identify early apoptotic cells, whereas cells in the late stage of apoptosis were detected by PI. Thus, viable cells in the early stage of apoptosis were distinguished from late stage apoptotic cells and dead cells by double labeling with annexin V and PI. U251MG, U87MG and MGG23 cells treated with the (P)RR Ab showed an increase in the proportion of dead cells (**Figure 5A**). Similar data were obtained by the caspase-3 assay. Treatment with the (P)RR Ab significantly increased caspase-3 activity in U251MG cells (the mean relative caspase 3 activity was 1.0 ± 0.3 *vs*. 1.8 ± 0.4, P = 0.01, n = 3) and MGG23 cells (1.0 ± 0.01 *vs*. 1.72 ± 0.06, P = 0.03, n = 3, **Figure 5B**). No significant difference was observed in U87MG cells (1 ± 0.04 *vs*. 1.06 ± 0.03, P = 0.1, n = 3). These results demonstrated that the (P)RR Ab induced apoptosis of glioblastoma.

(P)RR Ab suppresses cell invasion and migration

The effect of the (P)RR Ab on cell invasion and migration was investigated using the wound healing assay in U251MG and U87MG cells. Compared with that of the control treatment, wound closure in (P)RR Ab-treated cells was attenuated significantly in U251MG cells [the average percentage of wound closure was 45 ± 1.9% *vs.* 26 ± 2.9% 24 h after scratching (P = 0.02, n = 5), and 70 ± 2.2% *vs.* 40 ± 4.7% 48 h after scratching (P = 0.02, n = 5), **Figure. 6A**] and U87MG cells [the average percentage of wound closure was 22.2 ± 6.4% *vs.* 18.6 ± 7.4% 24 h after scratching (P = 0.6, n = 5), and 75.6 ± 9.5% *vs.* 47.3 ± 6.2% 48 h after scratching (P = 0.004, n = 5), **Figure. 6B**].

The effect of the (P)RR Ab in glioma stem-like cells derived from U87MG and U251MG was investigated. Glioma stem-like cells were plated on low-attachment 96-well plates. Cells were cultured in serum-free stem cell medium and then treated with the (P)RR Ab. Four days after treatment, control cells having protrusions were partially attached on the bottom of the plate. In contrast, all cells treated with the (P)RR Ab began to form spheres and float (**Supplementary Fig. S4**). Thus, the results clearly indicated that the (P)RR Ab suppressed cell adhesion.

In summary, this study showed that the monoclonal (P)RR Ab suppresses gliomagenesis characterized by cell proliferation, stemness and invasion (**Figure 7**).

Discussion

The present study has shown that the monoclonal (P)RR Ab inhibits gliomagenesis by inhibiting the activity of the Wnt signaling pathway. Our data have also indicated the potential contribution of (P)RR to stemness, based on the following observations. First, SOX2 expression positively correlated with (P)RR expression in human glioma tissues. Second, the expression of key molecules that are part of the Wnt signaling pathway and stemness markers were reduced significantly in cells treated with the (P)RR Ab. Third, treatment with the (P)RR Ab inhibited cell proliferation and larger sphere formation. Forth, the (P)RR Ab induced apoptosis and inhibited cell invasion and migration. Collectively, these data suggest that the (P)RR Ab is a novel promising molecular target drug for treating glioblastoma.

In several cancers including gliomas, activation of the Wnt signaling pathway promotes cell proliferation, migration and invasion, and inhibits apoptosis [29, 30]. Cruciat et al. [13] demonstrated that the extracellular domain of (P)RR interacts with Wnt components LRP6 and Frizzled to activate the Wnt signaling pathway. Our previous studies have indicated that (P)RR plays an important role in the development of glioma by activation of the Wnt signaling pathway [16]. The present study showed that treatment with a monoclonal (P)RR Ab significantly reduced proliferative activity of U251MG, U87MG and MGG23. Our data also showed that administration of the (P)RR Ab reduced the progression of tumor volume significantly in mice xenografted with U87MG cells. Recent studies have shown that a monoclonal (P)RR Ab suppresses cell proliferation by inhibiting the activation of the Wnt signaling pathway in pancreatic adenocarcinoma and colorectal cancer [18, 19]. Translocation of β-catenin into the nucleus following aberrant activation of the Wnt signaling pathway enhances cell proliferation through expression of Wnt target genes, such as c-myc and cyclin D1 [31, 32]. As observed for pancreatic adenocarcinoma and colorectal cancer, we found that treatment with the (P)RR Ab reduced the expression level of c-myc significantly. These data support the hypothesis that the inhibitory effects of the (P)RR Ab on cell proliferation of glioblastoma cells are caused by attenuating the activation of the Wnt signaling pathway. GSCs are thought to be precursors of a variety of malignant gliomas and a small subpopulation of tumor cells that self-renew and proliferate to maintain tumor growth [33, 34]. The Wnt signaling pathway has been reported to regulate self-renewal of GSCs, in addition to cell proliferation, migration and differentiation; potentially providing an opportunity for therapeutic targeting [35–37]. For example, antihelmintic drug Pyrvinum pamoate targeted for Wnt pathway [38], Resveratrol targeted for nuclear β-catenin and c-Myc [39] and Tankyrases targeted for Axin [40] have already been reported as representative inhibitors of Wnt signaling pathway. Furthermore, these therapies also reduce GSC tumorigenicity. (P)RR is located on the upstream of nuclear β-catenin, c-Myc and Axin in Wnt pathway, which implies that (P)RR Ab will have comprehensive pharmacological effects of these reagents.

Oct3/4, SOX2 and c-myc are linked to the formation of GSCs, carcinogenesis and tumor progression, which play pivotal roles in the maintenance of GSCs phenotype and stemness [41–45]. In the present

study, we focused on SOX2 expression, because increased SOX2 levels are associated with a poor outcome for glioma patients [46]. We previously found that (P)RR expression correlates positively with the malignancy of glioma regardless of the presence /absence of *IDH1*^{R132H} [16]. Our present data have also revealed that there is a positive relationship between (P)RR and SOX2 expression irrespective of *IDH* or 1p19q status in glioma patients. These data suggests that (P)RR and SOX2 expression is not affected by distinctive mutations of glioma but by grade. In addition, *in vitro* studies have shown that expression of Oct3/4, SOX2 and c-myc were reduced significantly in U251MG, U87MG and MGG23 cells by treatment with the (P)RR Ab. These data suggest that (P)RR expression affects these molecules responsible for stemness. Our data have also shown that in the MTT assay and the sphere formation assay using MGG23 cells as a human GSC cell line, that the (P)RR Ab suppressed cell proliferation and a large sphere formation significantly. These data support the concept that (P)RR regulates stemness in glioma and the (P)RR Ab inhibits biological roles of GSCs.

To further explore the molecular mechanisms by which the (P)RR Ab inhibits glioblastoma cell proliferation, we investigated whether apoptosis is induced by using flow cytometry and caspase-3 assays. Data indicated that treatment with the (P)RR Ab induced apoptosis in these cells. However, in caspase-3 assay using U87MG cells, the effect of (P)RR Ab was limited. Other apoptosis pathway may be involved in the effect of (P)RR Ab in U87MG cells. Data indicated that treatment with the (P)RR Ab induced apoptosis in these cells. Recent studies have indicated a possible relationship between glioblastoma invasion and the GSC subpopulation [47]. The activity of the Wnt signaling pathway may also contribute to invasiveness in many malignancies [35]. In our wound healing invasion assay, treatment with the (P)RR Ab suppressed cell invasion of U251MG and U87MG. Furthermore, treatment with the (P)RR Ab suppressed cell adhesion of U251MG and U87MG cultured under stem cell conditions. Taken together, these data suggest that the (P)RR Ab suppress glioblastoma through multiple mechanisms.

There are several limitations in the present study. The main limitation is the small sample size of patients and single institute retrospective design in IHC study. Second, the only one cell line was used in *vivo* study and further study using orthotopic intracranial models are required to examine whether therapeutic (P)RR antibodies pass through blood brain barrier. Third, safety of (P)RR Ab administration needs to be confirmed for clinical application. Future studies will be performed to overcome these limitations.

In conclusion, the present study has revealed that a monoclonal (P)RR Ab inhibited gliomagenesis such as cell proliferation, stemness and invasion. These data suggest that (P)RR is a novel therapeutic target via inhibition of stemness and the Wnt signaling pathway.

Materials And Methods

Glioma primary tissues

Glioma tumor specimens were obtained from patients who underwent a craniotomy at Kagawa University Faculty of Medicine between 2013 and 2019. Patients that underwent chemotherapy or radiotherapy before surgery were excluded from the study. Fifty-six patients were included for immunohistochemistry (IHC) analysis.

This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the institutional review board of Kagawa University Hospital (approval number 2020-118). The institutional review board of Kagawa University Hospital approved that the requirement for informed consent was waived in this study because patient identifiers were completely removed and data were collected retrospectively.

IHC for (P)RR and SOX2

Representative sections were prepared from the resected glioma samples. Tissues were fixed in 10% buffered formalin for 24–48 h and embedded in paraffin. Immunohistochemical staining was performed in an automated system using the Ventana BenchMark ULTRA (Roche Diagnostics, Basel, catalog #518-108496), according to the manufacturer's instructions. For the primary antibody, we used the anti-human (P)RR rabbit polyclonal antibody [20, 21] with a 1:6000 dilution, and the anti-SOX2 (sex determining region Y-box 2, SP76) rabbit monoclonal antibody (Cell Marque, Rocklin, CA, #371R-1) with a 1:100 dilution. Proportion score (PS) and intensity score (IS) were blindly scored by pathologists. PS was counted using the hotspot method, i.e., percentage areas of positive staining were calculated as the average of the three most strongly stained areas of one section. IS was based on staining intensity: 1 = weak, 2 = moderate and 3 = strong.

Cell culture

Human glioblastoma cell lines (U251MG, U87MG) were obtained from the American Type Culture Collection. These cell lines were incubated in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, catalog #11965118) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, catalog #7524-500ML) at 37 ℃ under 5% CO_2/95% air in a humidified incubator. The human glioblastoma derived cancer stem cell line, MGG23 cells, were provided by Dr. Hiroaki Wakimoto at Massachusetts General Hospital [22, 23]. MGG23 cells were cultured in neurobasal medium (Thermo Fisher Scientific, catalog #21103049) supplemented with L-glutamine (3 mM; Thermo Fisher Scientific, catalog #A2916801), B27 supplement (Thermo Fisher Scientific, catalog #17504044), EGF (20 ng/mL; R&D Systems, Minneapolis, MO, catalog #236-EG-200) and FGF (20 ng/mL; Peprotech, Cranbury, NJ, catalog #100-41) to establish neurosphere cultures enriched for GSCs.

Glioma stem-like cells were cultured and isolated from U251MG and U87MG glioma cell lines by using serum-free medium, as described above.

Generation of the (P)RR Ab

We used the monoclonal (P)RR Ab in both *in vitro* and *in vivo* studies. As shown previously, the (P)RR Ab was designed to target the extracellular domain of the (P)RR by using the rat lymph node method [19]. Control supernatant medium was used as the vehicle treatment.

Western blotting

Protein concentrations were measured using the Bradford method. Total protein extracts (30 μg) were electrophoretically separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Blots were blocked with blocking solution (LI-COR, Lincoln, NE, catalog #927-40000). The primary antibodies used (1:1000 dilution in blocking solution) were the anti-active-β-catenin (Millipore, Billerica, MA, catalog #05-665), c-myc (Santa Cruz Biotechnology, Dallas, TX, catalog #sc-40), SOX2 (Cell Signaling, Danvers, MA catalog #2748) and Oct (octamer-binding transcription factor) 3/4 (Santa Cruz Biotechnology, catalog #sc-5279). After incubation with secondary antibodies (1:1000 dilution in blocking solution) coupled with infrared dyes (IRDye 800 goat anti-rabbit immunoglobulin G [IgG], IRDye 680 goat anti-mouse IgG and IRDye 680 donkey anti-goat IgG), protein expression was detected using an Odyssey scanner (LI-COR, catalog #9141-00). The membranes were reprobed with an antibody for β-actin (Sigma-Aldrich, catalog #A5441) to confirm equal protein loading.

Cell proliferation assay

The water-soluble tetrazolium (WST)-1 assay was performed to determine the cell proliferation of U251MG and U87MG cell lines according to the manufacturer's instructions (Takara Bio, Shiga, catalog #MK400). First, 8×10^3 cells were seeded onto the wells of 24-well plates. After incubation for 72 h, treatment with the (P)RR Ab at 100, 200 and 400 μg/μL was performed. After a further 72 h, 50 μL of the WST-1 reagent was added to the 500 μL cell culture medium in each well and incubated for 1.5 h at 37 °C. Finally, the absorbance was measured at 450 nm by using a microplate reader (Corona Electric Co., Ltd, Ibaraki, catalog #SH-9000).

The MTT assay was performed using the MGG23 cell line. 1.5×10^4 cells per well were plated in 96-well plates and treated with the (P)RR Ab at 200 μg/μL. After 48 h, the MTT solution was added to each well and cells were incubated for 2 h at 37 °C. Thereafter, formazan was solubilized in absolute ethanol, and the absorbance was measured at 595 nm by using a microplate reader (Corona Electric Co., Ltd, Ibaraki, catalog #SH-9000).

Direct cell counting

Direct cell counting was performed using U251MG and U87MG. 8×10^4 cells were seeded onto the wells of 6-well plates. After incubation for 48 h, treatment with the (P)RR Ab at 100, 200 and 400 μg/μL was performed. After a further 48 h incubation, direct cell counting was performed to determine cell numbers. Then, the cells were treated with 0.25% trypsin-EDTA (Thermo Fisher Scientific, catalog #R001100), centrifuged and the cells resuspended. The cell suspensions were stained with AccuStain (NanoEnTek, Seoul, catalog #ADR-1000). Aliquots of the cell suspensions (20 μL) were then loaded into an AccuChip

4× Counting kit (NanoEnTek, catalog #AD4K-200) and viable cells (per mL) were counted using an automated cell counter (NanoEnTek, catalog #ADAM-MC).

In vivo tumorigenicity studies

Experimental protocols and animal care were performed according to the guidelines for the care and use of animals established by Kagawa University, and the principles of the Declaration of Helsinki. The animal Experimentation Ethics Committee at Kagawa University approved the experimental protocols (approval number: 2020-18648-2). Five week old male BALB/c (nu/nu) nude mice were purchased from CREA (Tokyo, Japan) and were maintained in specific pathogen free animal facilities under a controlled temperature (24 ± 2 °C) and humidity (55 ± 5%) with a 12-h light-dark cycle. Mice were bred by standard chow and water ad libitum. U87MG cells were grown in 15 cm^2 dishes, trypsinized and centrifuged to collect cell pellets after reaching a confluency of 70–80%. The pellets were diluted with PBS to a final concentration of 1×10^6 cells/200 µL. Cells were inoculated by subcutaneous injection into the right flank of mice previously anesthetized via intraperitoneal administration of ketamine (100 mg/kg)/xylazine (10 mg/kg). Mice were treated with the (P)RR Ab by intraperitoneal injection every three days over a 20-day period from the 3rd day after cell transplantation. Control supernatant medium was served as the vehicle treatment. Tumor growth was monitored by measuring the diameter of the local tumors at the implant site with a digital caliper every 5 days up to the end of the experiment. Tumor volumes were calculated according the formula: length × $width^2$ × 0.5.

Sphere formation assay

GSC spheres were enzymatically dissociated to single cells and placed in 96-well plates at an optimal density (1×10^4 cells) in nonadherent conditions and treated with the (P)RR Ab at 200 µg/µL. After 48 h, cells were imaged using an EVOS XL Core microscope (Thermo Fisher Scientific, catalog #12562751). ImageJ (National Institutes of Health) was used to analyze the size and quantity of spheres.

Detection of apoptosis using annexin V and propidium iodide

After treatment with the (P)RR Ab, U251MG and U87MG cells were incubated for 96 h, whereas MGG23 cells were incubated for 48 h. U251MG and U87MG cells were then treated with 0.25% trypsin-EDTA, centrifuged, washed twice in PBS and resuspended in annexin-binding buffer (1×) according to the manufacture's protocol (Nacalai tesque, Kyoto, catalog #15342-54). MGG23 cells were treated with TrypLE Express (Thermo Fisher Scientific, catalog #12604013). Next, the cells were stained with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI) working solution for 15 min at room temperature, after which 400 µL of annexin-binding buffer was added. Flow cytometry was performed and emissions at 525 nm (FITC) and 675 nm (PI) were monitored.

Detection of apoptosis by the Caspase-3 assay

Forty-eight hours after treatment with the (P)RR Ab, caspase-3 activity was detected according to the manufacture's protocol (Medical & Biological Laboratories, Nagoya, catalog #4800). Reaction buffer (2×) and the caspase-3 substrate were added to cell lysates, which were injected into the wells of 96-well plates and incubated at 37 ℃ for 8 h. The absorbance was then measured at 405 nm with a microplate reader (Corona Electric Co., Ltd, Ibaraki, catalog #SH-9000).

Wound healing assay

U251MG and U87MG cells were grown to confluence in 12-well plates to evaluate cell invasion by the wound healing assay. A thin "wound" was introduced by scratching the monolayer with a sterile 200 μL pipette tip. Wells were washed with PBS to remove dead cells and debris, and the medium was replaced with serum-free medium for culturing. Cells were treated with the (P)RR Ab. Migration of cells into the gap was imaged over hours. The wound gap was measured and expressed as a percentage of wound closure. Five gap distances were randomly measured using ImageJ software.

Statistical analysis

Statistical analyses were performed with EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). Univariate analysis was conducted using the Mann-Whitney U test or unpaired t-test for parametric data. Spearman's correlation coefficient was used to assess association between variables. $P < 0.05$ was considered significant.

Declarations

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Author contributions

The authors have made the following declarations regarding their contributions: T.F., Y.Sh., D.O., K.K., and A.N. designed this study. T.F., Y.Sh, T.K., K.S., R.I. and T.M. acquired the data. T.F. Y.Sh. and D.O. performed the analysis and interpretation of data. T.F., Y.Sh.wrote the paper. Y.Sh., D.O., K.K., K.M., A.N. and T.T. revised manuscript. T.K., K.S. and R.I. contributed reagents/ materials/ analytical tools.

Data availability statement

On reasonable request, derived data supporting the findings of this study are available from the corresponding author.

Additional information

Competing Interests: All authors have no conflicts of interest.

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Figures

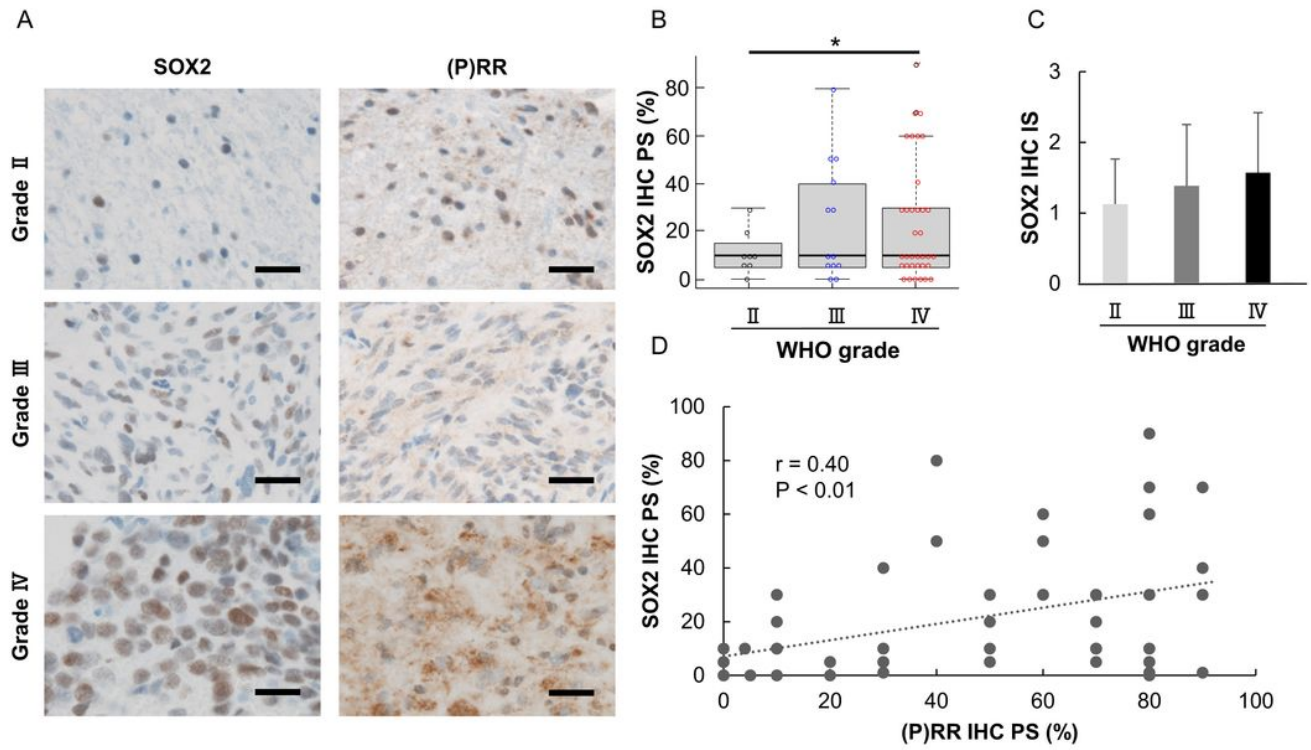

Figure 1

(P)RR and SOX2 expression in human gliomas. (A) Expression of SOX2 and (P)RR in representative tumor samples of each WHO grade by immunohistochemistry (scale bar = 50 μm). (B) PS and (C) IS of SOX2 expression increased according to the WHO grade. (D) SOX2 expression weakly correlated with (P)RR expression ($r = 0.40$, $P < 0.01$). *: $P < 0.05$.

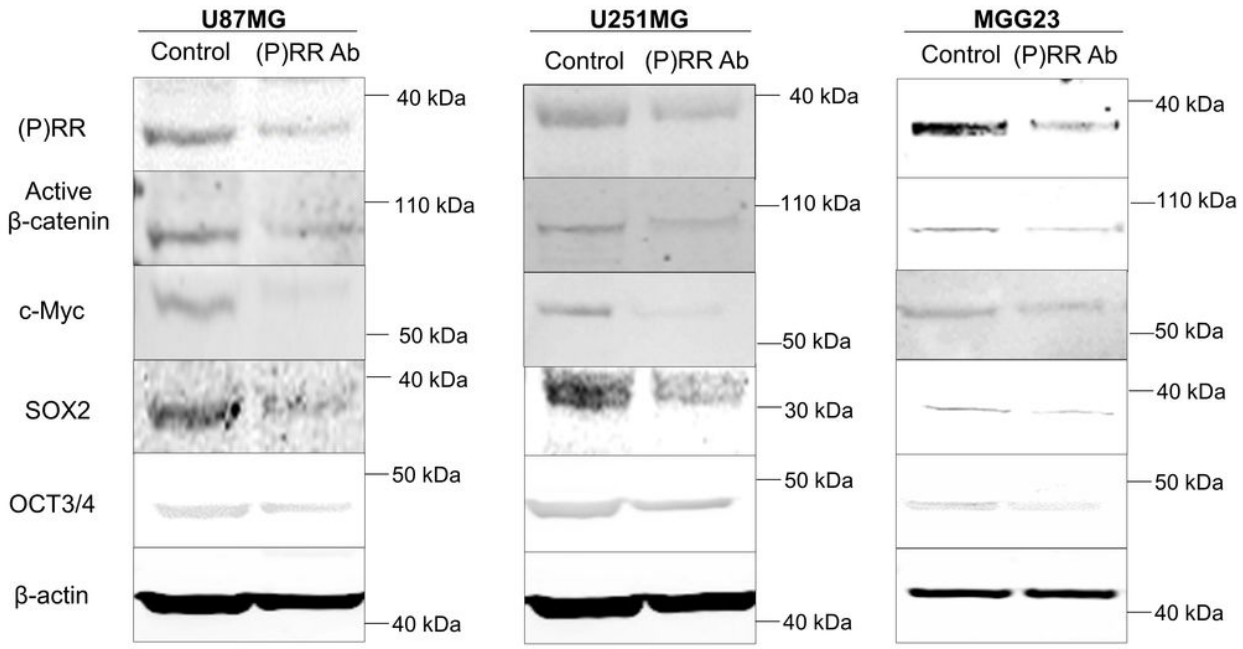

Figure 2

Effect of the (P)RR Ab on Wnt signaling and stemness markers. (A) Representative western blot images showing that treatment with the (P)RR Ab reduced the activity of the Wnt/β-catenin signaling pathway [active β-catenin and c-myc] and expression of stemness markers (SOX2 and Oct3/4) in U251MG, U87MG and MGG23 cells.

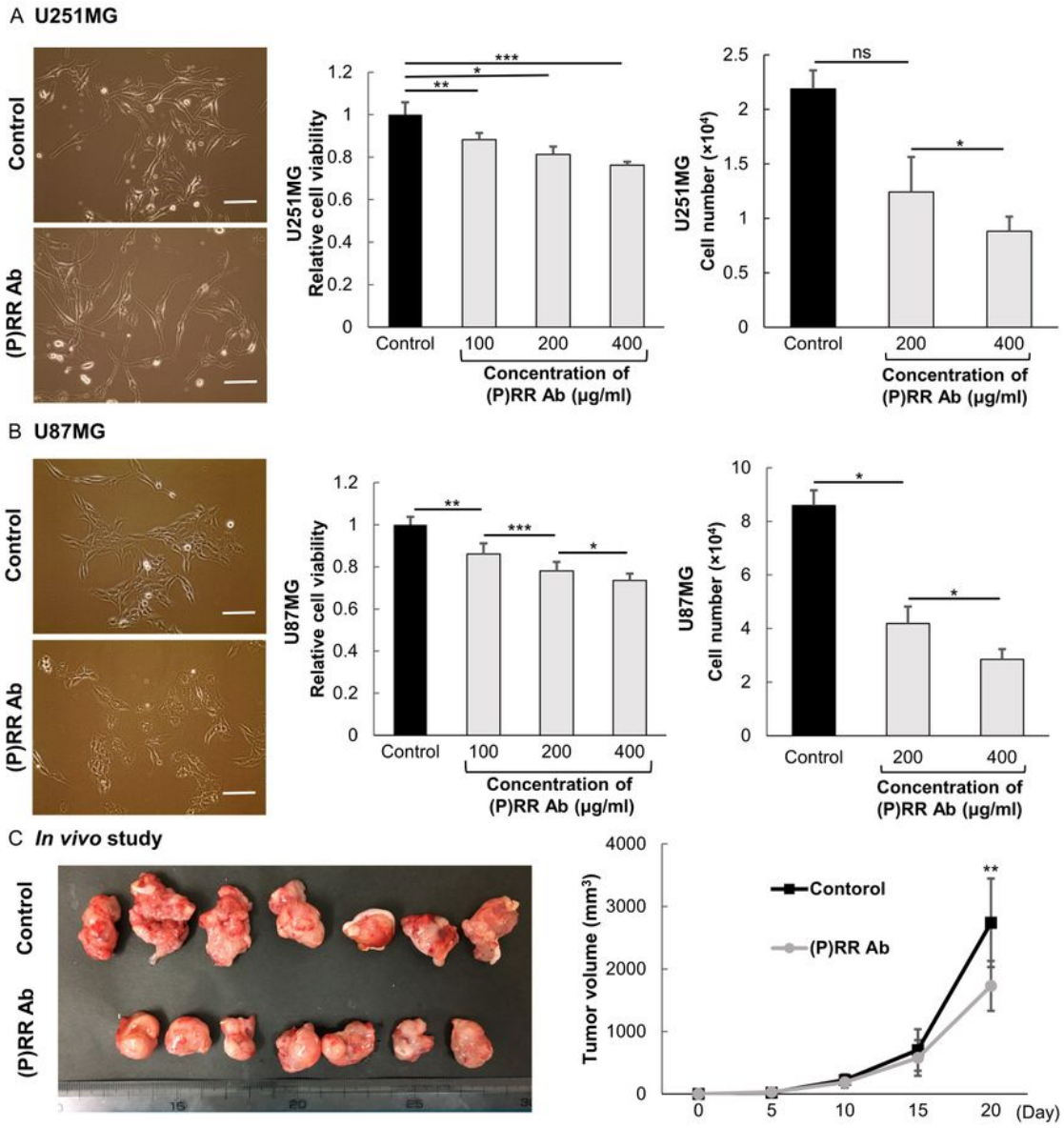

Figure 3

Antiproliferative effects of the (P)RR Ab on human glioblastoma cell lines *in vitro* and *in vivo*. The (P)RR Ab dose-dependently reduced cell proliferation in WST-1 (n = 3) and cell numbers (n = 4) in (A) U251MG cells and (B) U87MG cells (scale bar = 200 μm). (C) *In vivo* antitumor effect of systemic administration of the (P)RR Ab on engrafted tumors of U87MG cells in athymic nude mice 20 days after injection (*left*), and

time dependent changes in tumor volume (*right*). The mean tumor volume after 20 days was 1730 ± 399 mL *vs*. 2739 ± 707 mL ($P < 0.01$, $n = 7$). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

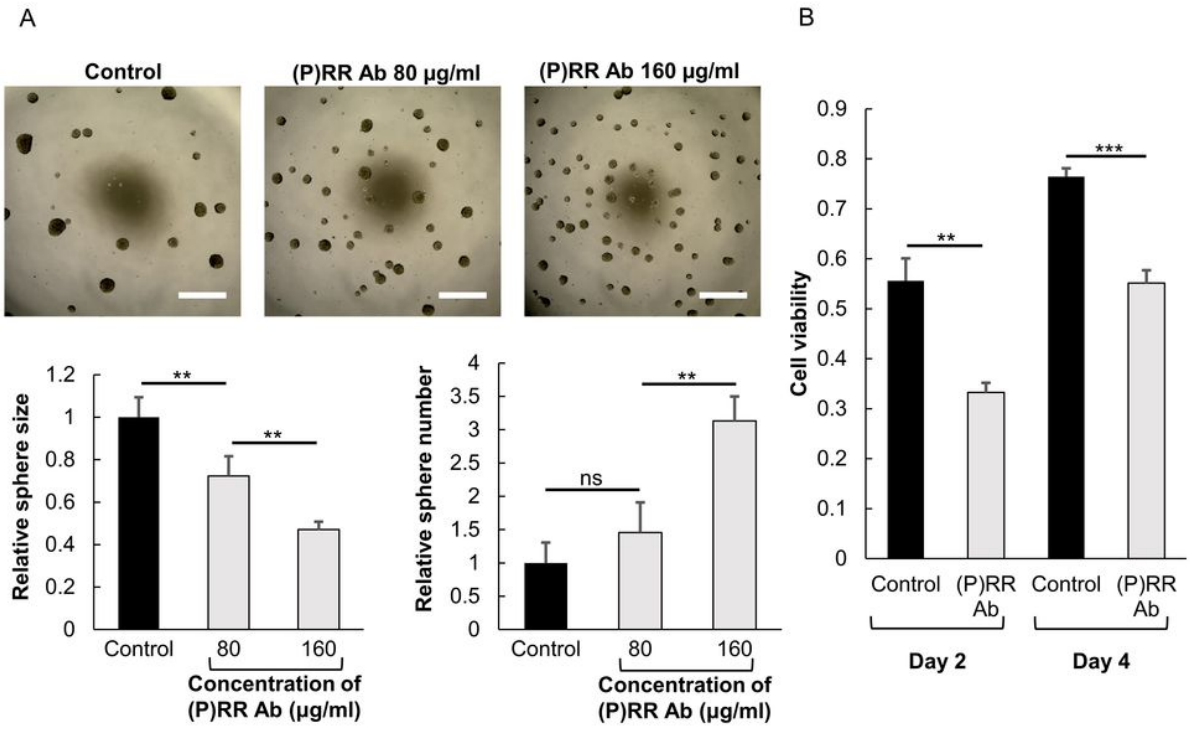

Figure 4

Antiproliferative effects of the (P)RR Ab on the human glioma stem cell line. (A) Dose-dependent changes in sphere formation of MGG23 after treatment with the (P)RR Ab (scale bar = 75 µm). The sphere size decreased dose-dependently, while the quantity of the spheres increased ($n = 4$). (B) The (P)RR Ab reduced cell proliferation in the MTT assay, both dose-dependently and time-dependently ($n = 4$). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

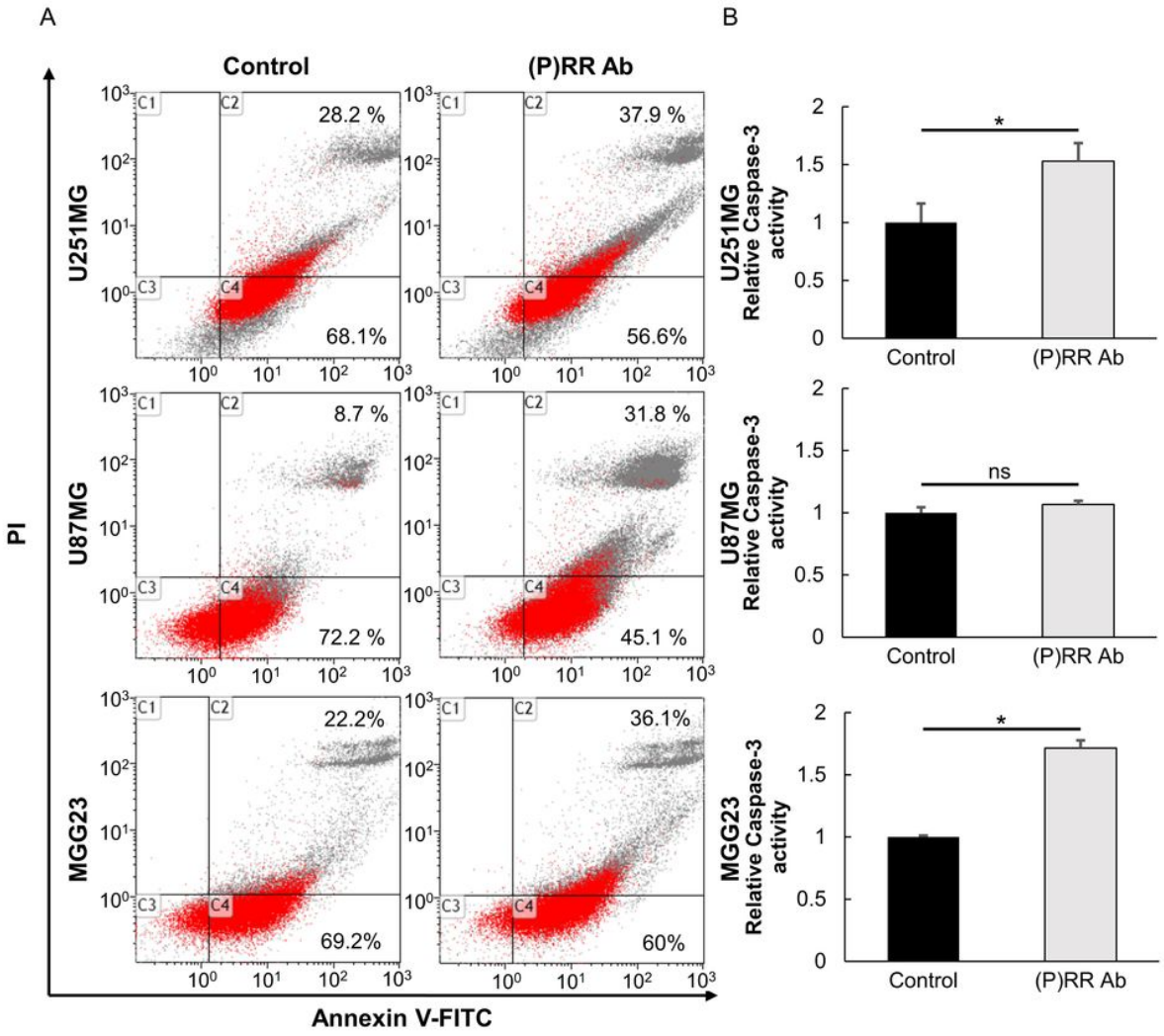

Figure 5

The (P)RR Ab induces apoptosis of glioblastoma cell lines and the glioma stem cell line. (A) Detection of early and late apoptosis in U251MG, U87MG and MGG23 treated with control *(left)* or the (P)RR Ab (*right*). Cells were stained with FITC-labeled annexin V and PI, and the intensity of fluorescence was assessed at 525 nm (FITC) and 675 nm (PI). C1: dead cells; C2: late-staged apoptosis; C3: viable cells; C4: early-staged apoptosis. An increase in the proportion of dead cells was observed. (B) Caspase-3 activity in U251MG, U87MG and MGG23 cells treated with the (P)RR Ab is shown. U251MG and MGG23 cells treated with the (P)RR Ab increased caspase-3 activity significantly ($n = 3$). *: $P < 0.05$.

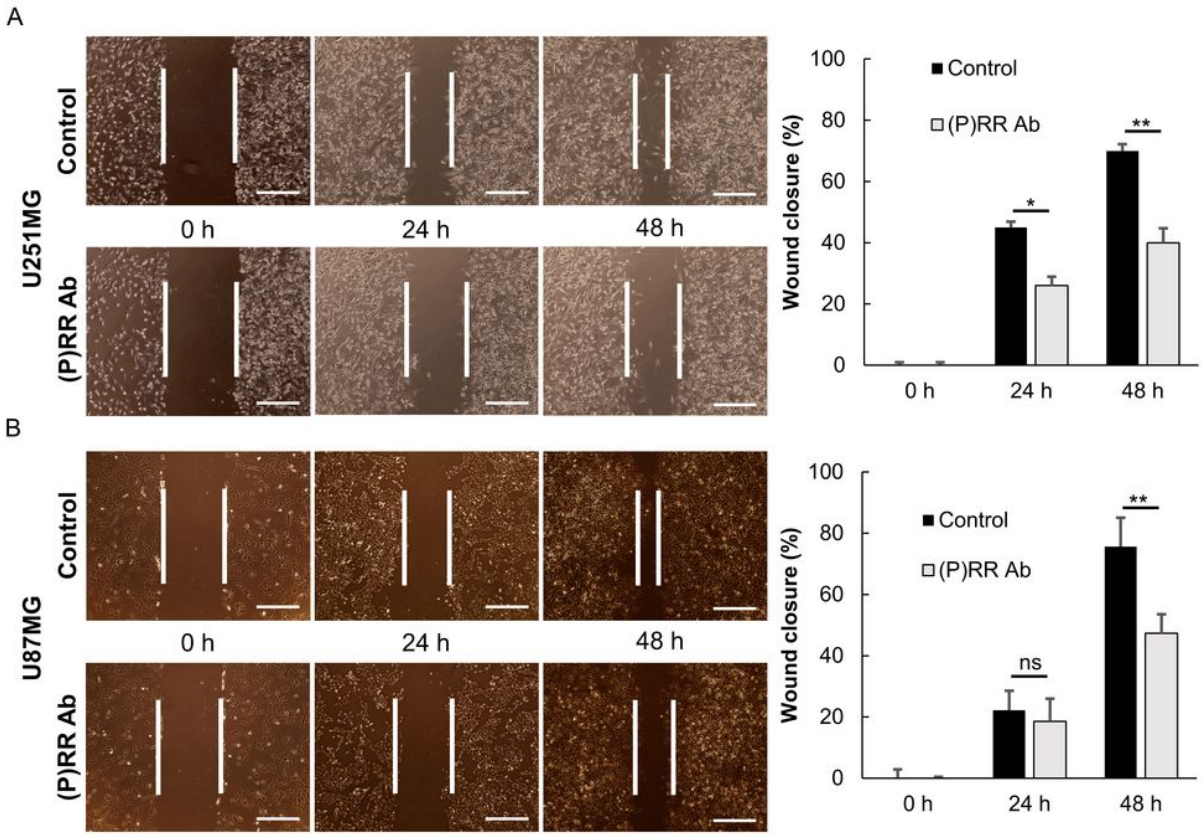

Figure 6

Effects of (P)RR Ab silencing on the invasion and migration of glioblastoma cell lines. Migration and invasion were evaluated in (A) U251MG and (B) U87MG by the wound healing assay. Representative images were captured at 0, 24 and 48 h after scratching. (scale bar = 75 μm). The white line shows the borders created by the original scratch. A graphical percentage of the wound closure in (A) U251MG and (B) U87MG is shown on the right side. The (P)RR Ab decreased cell invasion and migration significantly in U251MG and U87MG (n = 5). *: $P < 0.05$; **: $P < 0.01$.

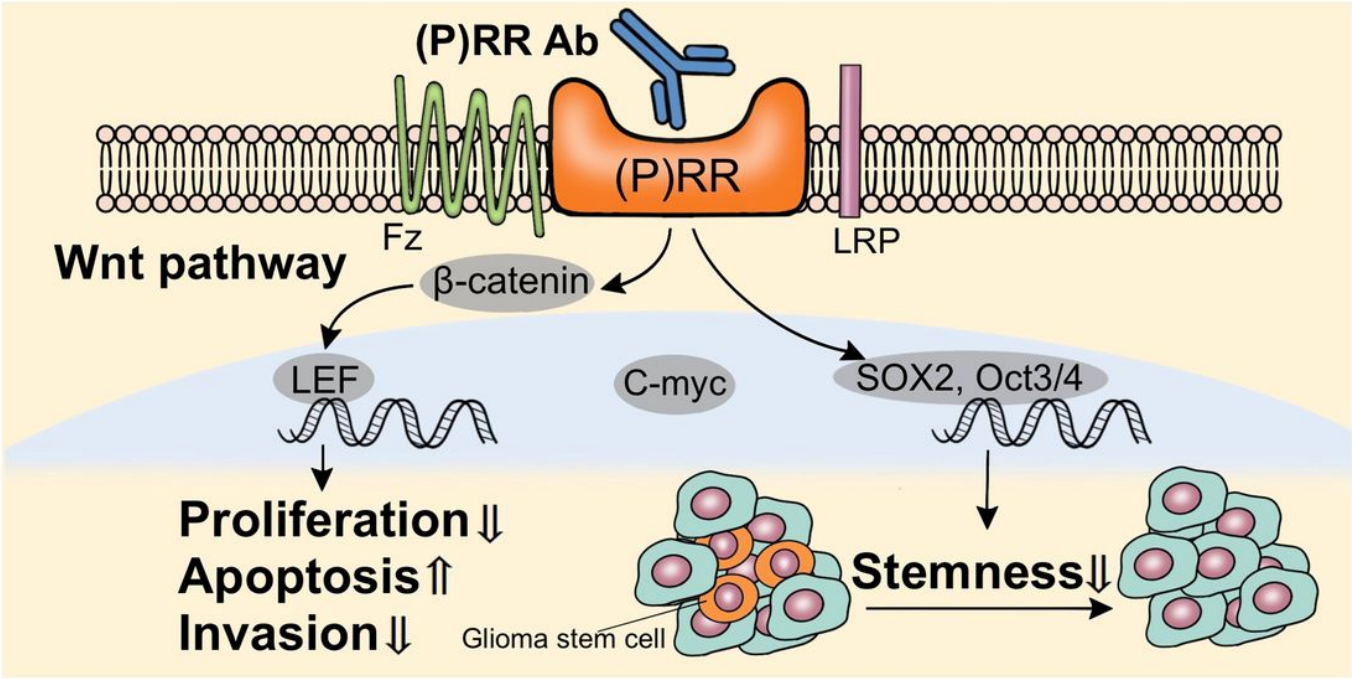

Figure 7

Proposed model for the effect of the (P)RR Ab in glioblastoma. Treatment with the (P)RR Ab decreases cell proliferation, invasion and migration and induces apoptosis via inhibition of the Wnt signaling pathway. The (P)RR Ab suppresses SOX2 and Oct3/4 expression and cell proliferation of the glioma stem cell line, which indicates that the (P)RR Ab inhibits stemness of glioma. Fz: Frizzled.

Supplementary Files

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