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Research Article

Wnt inhibition is dysregulated in gliomas and its re-establishment inhibits proliferation and tumor sphere formation

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ABSTRACT

Evidence indicates that the growth of glioblastoma (GBM), the most common and malignant primary brain cancer, is driven by glioma stem cells (GSCs) resistant to current treatment. As Wnt-signaling is pivotal in stem cell maintenance, we wanted to explore its role in GSCs with the objective of finding distinct signaling mechanisms that could serve as potential therapeutic targets. We compared gene expression in GSCs ($n=9$) and neural stem cells from the adult human brain (ahNSC; $n=3$) to identify dysregulated genes in the Wnt signaling pathway. This identified a six-gene Wnt signature present in all nine primary GSC cultures, and the combined expression of three of these genes (*SFRP1*, *SFRP4* and *FZD7*) reduced median survival of glioma patients from 38 to 17 months. Treatment with recombinant SFRP1 protein in primary cell cultures downregulated nuclear β-catenin and decreased *in vitro* proliferation and sphere formation in a dose-dependent manner. Furthermore, expressional and functional analysis of SFRP1-treated GSCs revealed that SFRP1 halts cell cycling and induces apoptosis. These observations demonstrate that Wnt signaling is dysregulated in GSC, and that inhibition of the Wnt pathway could serve as a therapeutic strategy in the treatment of GBM.

1. Introduction

Glioblastoma (GBM) is the most common and malignant primary brain tumor in humans. The tumor invariably recurs despite aggressive treatment, including surgery, irradiation and chemotherapy. Median survival is therefore less than one year in unselected patient populations. GBMs contain glioma stem cells (GSCs) that have a phenotype similar to adult human neural stem cells (ahNSCs) [1]. These cells are drivers of GBM propagation [2,3] and therapy resistance [4], and are thus believed to be responsible for the invariable recurrence of the tumor.

When cultured under serum-free, growth factor-enriched conditions, both ahNSCs and GSCs grow in spherical aggregates of cells known as neuro- or tumorspheres. Such spheres may be differentiated into the different lineages of the central nervous system [1,5]. The ability to form spheres has been shown to be an independent predictor of clinical outcome [6,7]. When transplanted to rodents, GSCs give rise to rapidly growing invasive tumors that resemble their tumor of origin [2].

The Wnt signaling system is a set of highly conserved pathways required for stemness and self-renewal in both embryonic and adult stem cells [8]. In Wnt/β-catenin signaling, β-catenin is phosphorylated by the β-catenin destruction complex (consisting of glycogen synthase kinase 3β (GSK3β), adenomatosis polyposis coli (APC), Axin and casein kinase 1 (CKI)) in the absence of Wnt activation. Phosphorylation at the N-terminal marks β-catenin for ubiquitination and degradation by the proteasome, thus keeping its levels low [9]. In humans, Wnt/β-catenin signaling is initiated when a Wnt ligand (19 in total) binds to the surface receptors Frizzled (Fzd; 10 in total) in the presence of the co-receptor low-density-lipoprotein-related protein 5/6 (LRP5/6). This induces phosphorylation of dishevelled (DVL), leading to the recruitment of the destruction complex to the Wnt-receptor complex, where it is inactivated. In the absence of the destruction complex unphosphorylated, active β-catenin accumulates and translocates to the nucleus where it activates transcription of target genes with the

Abbreviations: ahNSC, adult human neural stem cell; GSC, glioma stem cell

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help of nuclear transcription factors T-cell factor (TCF)/lymphoid enhancer factor (LEF) and their co-factors [10,11]. Wnt/β-catenin typically induces the transcription of genes involved in differentiation, proliferation and resistance to apoptosis [12,13].

Wnt signaling is modified by a number of antagonists, including the family of soluble Frizzled-related proteins (SFRPs). These are extracellular proteins that can bind either to the extracellular domain of Fzd receptors or directly to Wnts. They generally act as Wnt-inhibitors, although they also may enhance Wnt-signaling under special circumstances [14]. SFRP1, the most extensively characterized family member, is a well-established tumor-suppressor, and epigenetic silencing of SFRP1 occurs in a number of malignancies [15], including gliomas [16].

Aberrant Wnt-signaling is thought to be essential for cancer stem cells (CSCs) of various origins, including bladder, blood, breast, colon, and skin [8], where it regulates diverse processes involved in stem cell maintenance [8] and therapy resistance [17,18]. Unlike a number of other cancers, mutations in the Wnt pathway that result in constitutively active signaling are infrequent in GBM. Even so we recently found that Wnt has a key role among dysregulated pathways in GSCs [19]. Also, the expression and nuclear localization of β-catenin, and its transcription factor TCF4, is significantly higher in glioma tissue compared to normal brain tissue, and these findings also positively correlates to WHO glioma grade [20]. Moreover, oncogenic activities, such as proliferation, inhibition of apoptosis and invasion, have also been associated with Wnt/β-catenin signaling in glioma cell lines [20,21]. Only a few studies, however, have been performed on primary- and GSC cultures. Moreover, there are diverging reports regarding what ligands, receptors and mechanisms are responsible for Wnt signaling in GBM and GSCs.

GSCs share a number of characteristics and signaling pathways with ahNSCs [5,19,22]. We have previously identified the Wnt pathway as a distinguishing factor between normal and malignant stem cells from the adult human brain [19]. Here we present a more extensive analysis of the role of Wnt in GSCs and show that restoration of Wnt inhibition with the previously identified candidate SFRP1 reduces tumorigenicity through modification of p53- and cell cycle signaling. Together, these findings suggest that Wnt plays a pivotal role in GSC malignancy, and that Wnt pathway inhibition could provide a supplement to current GBM treatment.

2. Materials and methods

2.1. Biopsies and cell culturing

Biopsy specimens were obtained from 11 informed and consenting patients undergoing surgery for GBM and from seven patients operated for medically intractable temporal lobe epilepsy. Tissue harvesting was approved by the Norwegian Regional Committee for Medical Research Ethics, and histopathological diagnosis and grading was performed by a neuropathologist according to the WHO classification. Biopsies were kept in ice-cold Leibowitz-15 medium (L-15, Invitrogen) until isolation. Cells were isolated mechanically and enzymatically with trypsin-EDTA (Invitrogen), then blocked using 2 mg/mL human albumin (Octapharma Pharmazeutika Produktionges) and washed in L-15 twice. The cells were cultured in serum free Dulbecco's Modified Eagle's Medium (DMEM; Gibco) containing 10 ng/mL bFGF, 20 ng/mL EGF (both from R&D Systems) penicillin/streptomycin 100 U/mL of both (Lonza), heparin 1 ng/mL (Leo Pharma), HEPES 8 mM (Lonza) and 1:50 B27-supplement (Gibco), as previously described [6]. The cell culture assays were performed using three primary cell cultures (T2609, T0836 and T1008) between passage two and 10. We have previously shown that primary GSC cultures treated this way make invasive tumors upon transplantation [6], and that both ahNSCs and GSCs self-replicate and can be differentiated into the different lineages of the CNS [1]. In experiments using recombinant human SFRP1 (Sino Biological Inc., Thermo Fisher), the protein was added in concentrations between 0.8 and 3.2 μg/ml.

2.2. Proliferation assay

Cells were plated at a density of 2000 cells per well in a 96-well plate for suspension cells (Sarstedt) and cultured for 14 days with or without recombinant human SFRP1. Proliferation was subsequently assessed using Cell Proliferation Kit II XTT (Roche). The cells were incubated with the XTT solution for 18 h before absorbance was analyzed using a microplate reader.

2.3. Sphere formation assay

Cells were plated at a density of 500 cells per well in a 96-well plate for suspension cells (Sarstedt) and cultured for 14 days with or without recombinant human SFRP1. Sphere formation was subsequently counted using an automated colony counter (Gelcount, Oxford Optronics).

2.4. Apoptosis assay

Cells were plated at a density of 10,000 cells per well in V-shaped 96-well plates (Sarstedt) and cultured for 48 h with or without recombinant human SFRP1 (3.2 μg/ml). Apoptosis was subsequently measured using Cell Death Detection ELISA^{PLUS} (Roche).

2.5. RNA extraction and quantitative real time PCR

Total RNA was extracted from primary cell cultures at passage two using Qiazol and the RNeasy Micro Kit (both from Qiagen GmbH). RNA concentration was determined using a Nanodrop spectrophotometer (Thermo Fisher) and analyzed for quality using the Experion System (Bio-Rad). Only samples with an RNA quality indicator score > 8.0 were included for further analysis. Quantitative real time PCR (qPCR) was primarily performed using predesigned Low Density Array (LDA) cards (Applied Biosystems, Thermo Fisher), but was also performed individually for certain genes. The High capacity cDNA Reverse Transcription Kit, TaqMan Universal PCR Master Mix, TaqMan oligonucleotide primers and probes and the ABI Prism Detection System and software (all from Applied Biosystems) were used according to the manufacturer's instructions. Human *β-Actin* (TaqMan endogenous control reagents, Applied Biosystems) was used as housekeeping gene. The thermal cycling conditions for both methods were 2 min at 50 °C and 10 min at 94.5 °C, followed by 40 cycles of 30 s at 97 °C and 1 min at 59.7 °C. The relative gene expression levels were calculated using the $2^{-\delta\delta CT}$ method [23]. For interpretation of the results, high expression was defined as expressed $\delta Ct < 11$ in one or both groups (GSCs and/or ahNSCs), intermediate expression as δCt 11–15 in one or both groups and low expression/not expressed as $\delta Ct > 15$ in either group.

2.6. Microarray analysis

RNA samples were run in technical triplicates on a HumanHT-12 chip (Illumina). Analysis and statistics were performed using J-Express (Molmine). Differential gene analysis was carried out using RankProd [24]. Enriched pathways were identified using Webgestalt (Vanderbilt) and obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.7. DNA promoter methylation analysis

DNA was isolated using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). Methylation of the *SFRP1* promoter sequence was analyzed using a *SFRP1* specific probe and the EpiTect Methyl II PCR Assay System, all purchased from SABiosciences (Qiagen) and used according to the manufacturer's instructions.

2.8. Western blot

Cells were cultured for 10 days with or without recombinant human SFRP1 (3.2 μg/ml), then washed either in PBS (LONZA) and homogenized in 10 mmol/L Tris–HCl (pH 7.4), 1% SDS, 10 mmol/L NaF, and 2 mmol/L Na_3VO_4 or lysed using Nuclei EZ Prep (Sigma-Aldrich) for extraction of nuclei. Total protein content was measured using the BCA Protein Assay Kit (Pierce, Thermo Fisher) and samples were stored at −70 °C. Thawed aliquots (30 μg of total protein/aliquot) were mixed with 25% 4XLDS sample buffer (Clear Page) and 5% mercaptoethanol (Sigma-Aldrich) and boiled for 5 min at 100 °C. The samples were separated on a gradient gel (4–12% Clear Page SDS gels) and transferred to 0.45 μm PVDF membranes (Amersham) by electroblotting (100 V, 1 h at room temperature). For immunoblotting, the PVDF membranes were first incubated with blocking buffer (5% dry milk (Bio-Rad) in TBS containing 0.1% Tween-20 (TBST)) for 1 h at room temperature. The membranes were then washed 3 × 5 min with TBST and incubated with primary antibody (mouse anti-active β-catenin (anti-ABC), 1:1000 from Millipore; Merck KGaA) in blocking buffer overnight at 4 °C. Next, the membranes were washed 3 × 5 min in TBST and incubated with HRP-conjugated secondary antibody (Donkey anti-mouse IgG, 1:10,000; Amersham) for 1 h at room temperature. Finally, membranes were rinsed several times in wash buffer, incubated with LumiGLO Reserve CL substrate Kit (KPL) for 1 minute and scanned using the Kodak Image Station 400 MM PRP (Kodak).

2.9. Immunocytochemistry

Spheres were fixed in paraformaldehyde, cryoprotected and incubated in OCT (Tissue-TEK). Blocks were then cryosectioned at 10 or 20 μm on a freezing microtome, and thawed onto Super Frost/Plus microscope slides (Menzel-Gläzer). Sections were washed, blocked and incubated overnight at 4 °C with primary antibody: anti-ABC (1:200) from mouse (Millipore) and anti-glial fibrillary acidic protein (GFAP; 1:1000) from rabbit (DAKO). Secondary antibodies were anti-mouse Alexa 488 (donkey, 1:500; Invitrogen) and anti-rabbit Cy3 (donkey, 1:800; Jackson Laboratories). Hoechst 33258 (1:5000; Sigma) was used for nuclear staining. Analysis and image acquisition was performed on an Olympus BV 61 FluoView confocal microscope, using the FV10-ASW 1.7 software (Olympus).

2.10. ELISA analysis of conditioned medium

Cells were plated at a density of 5000 cells per well in a 96-well plate for suspension cells (Sarstedt). The standard protocol was used for the GSC cultures, whereas for the ahNSC cultures bFGF was exchanged with 20 ng/mL TGF-α (R&D systems) and supplemented with 1% fetal bovine serum (PAA Laboratories). Quantification of SFRP1 protein was performed on technical duplicates from aspirated culture medium using the ELISA Assay Kit for SFRP1 (USCN Life Science Inc.).

2.11. Cell cycle analysis

Cells were cultured for 10 days with or without recombinant human SFRP1 (3.2 μg/ml). During the last 24 h samples were incubated with EdU (10 μM) from the Click-iT EdU Alexa Fluor 488 Cell Proliferation Assay Kit (Invitrogen). The analysis was assessed using an LSRII flow cytometer (BD Biosciences) and performed twice as independent experiments.

2.12. Survival analysis

For the correlation between the Wnt signature expression and clinical outcome, clinical data was extracted from the Rembrandt database. Only glioma patients with known clinical information and gene expression data were included. A Cox proportional hazard regression model was fitted to the data. The variable selection for this model was performed in a forward, stepwise manner with Akaike information criterion. From the estimated regression model, a score was calculated as the exponentiated regression equation. The patients were divided into two groups based on this score by hierarchical clustering, these groups were then compared in a Kaplan–Meier plot and the *p*-value of the corresponding log rank test was calculated.

2.13. Statistics

Data are presented as ± standard deviations. Differences were assessed by two-tailed Fisher's exact test and unpaired two-tailed Student's *t*-test (Excel, Microsoft Office) based on three independent experiments. Statistical significance was determined at *p*-values < 0.05 (*) and < 0.01 (**).

3. Results

3.1. Regulation of key Wnt signaling genes in GSCs

Both GSCs from primary GBMs ($n=9$) and ahNSCs from temporal lobe resections ($n=3$) were cultured as spheres, and upon transplantation to mice the GSCs formed invasive tumors (data not shown). Furthermore, both cell types expressed typical stem cell markers (see Supplementary data). To further explore the role of Wnt in GBM we performed a qPCR based expressional comparison of Wnt-related genes. Overall, the similarities were greater than the differences as most genes, including the gene encoding β-catenin (*CTNNB1*), were expressed at the same level in the two cell types (Fig. 1). However, several important Wnt signaling genes were differentially regulated. Eight Fzd receptors were highly expressed in ahNSCs and/or GSCs. Two of these, *FZD7* and *FZD3*, were significantly upregulated in GSCs, eight and 10 times, respectively (Figs. 1 and 2A). The Wnts were divided into eight intermediately expressed (Fig. 2B) and three highly expressed genes (Fig. 2C). Among the highly expressed, two were significantly regulated: *WNT5B* was 15 times downregulated and *WNT7A* 67 times upregulated in GSCs (Figs. 1 and 2C). Of the soluble Wnt inhibitors, three were highly expressed in ahNSCs. Two of these were significantly regulated: *SFRP4* was 15 times upregulated and *SFRP1* was 360 times downregulated in GSCs (Figs. 1 and 2D). We also examined the expression of nuclear transcription factors and found that four were highly expressed, among which *TCF3* and *LEF1* were upregulated in the GSCs, nine and 10 times, respectively (Figs. 1 and 2E).

3.2. Identification of Wnt signature that impacts prognosis

Interestingly, the pattern of upregulation of *FZD7*, *FZD3*, *SFRP4* and *WNT7A*, and downregulation of *WNT5B* and *SFRP1*, was present in all nine tumors, thus constituting a signature of Wnt initiating genes in GSCs (Fig. 3A). To investigate the association

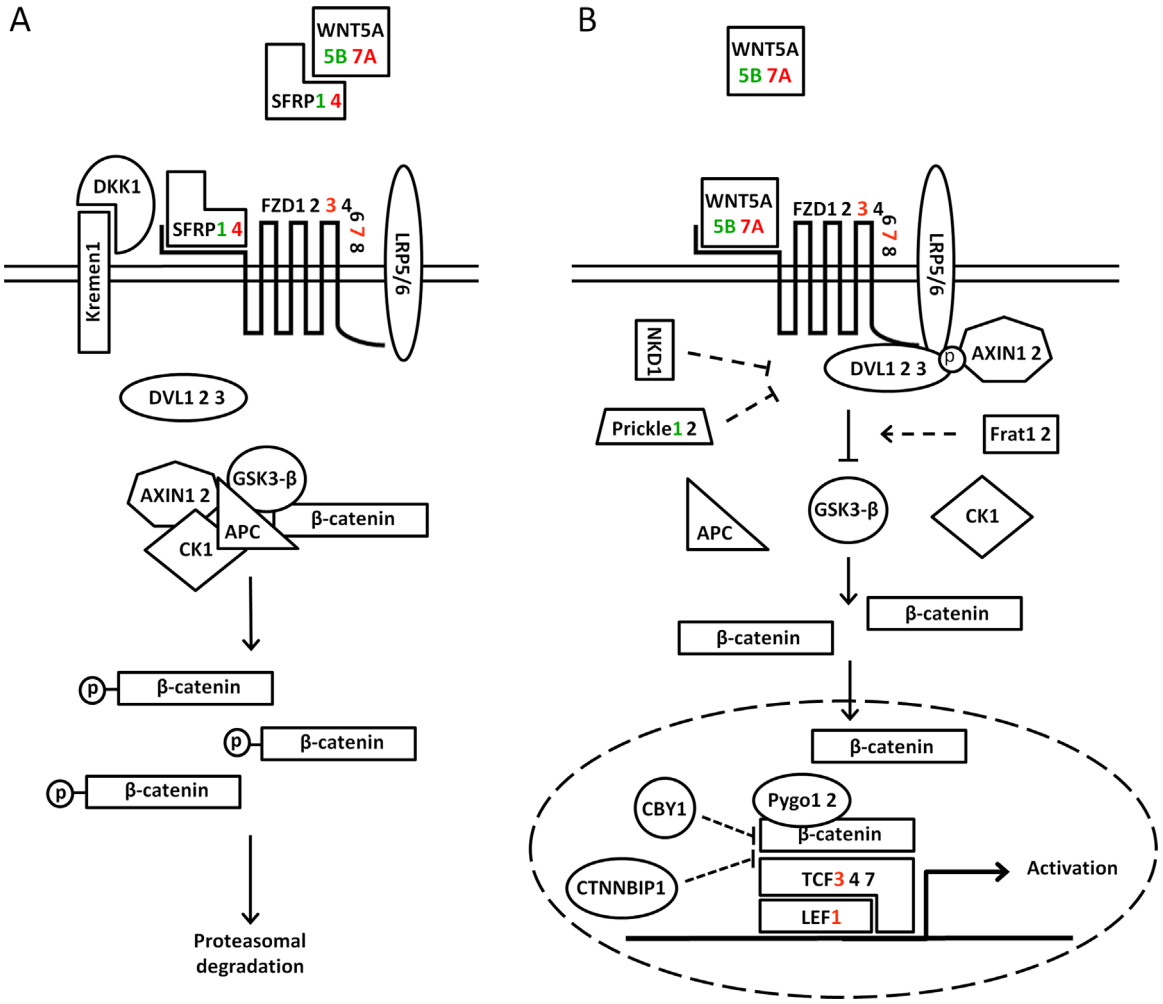

Fig. 1. Pathway map representing the core common Wnt signaling mediators that are highly expressed in ahNSCs and GSCs. Where a gene is significantly regulated ($p < 0.05$), green indicates downregulation in GSCs and red indicates upregulated in GSCs. (A) The unstimulated state. (B) The stimulated state.

between the Wnt signature and clinical outcome in glioma patients we examined the impact of these genes on patient survival (Supplementary Fig. S1). We found that the combined downregulation of *SFRP1*, and upregulation of *SFRP4* and *FZD7*, was associated with a reduction in median survival from 38.3 months to 17.0 months ($p < 0.00001$; Fig. 3B).

3.3. *SFRP1 is downregulated through promoter methylation*

Wnt signaling consists of an interplay between several distinct pathways, and the function and relative importance of the individual pathways in glioma remains largely unknown. With the exception of WNT3A and WNT5A, it has proved difficult to identify Wnt ligands or receptors as selective for any part of the Wnt pathway. Thus, in order to comprehensively manipulate this pathway we chose to further explore the role of the universal Wnt-inhibitor SFRP1, the only gene from the signature known to be capable of blocking all the different Wnt-pathways. We compared its expression in GSCs, ahNSCs and normal brain and found that it was downregulated in GSCs compared to both ahNSCs and normal brain tissue (Fig. 4A). To investigate the protein level of SFRP1 we quantified SFRP1 protein in cell medium using ELISA detection. Three ahNSC cultures and four GSC cultures were incubated for seven days and conditioned medium was aspirated and analyzed. Whereas the ahNSC cultures contained 25.3 ± 0.4 ng/ml SFRP1, all four GSC cultures were below the detection limit (< 0.05 ng/ml; $p < 0.000001$; Fig. 4B).

Next we performed an analysis of the SFRP1 promoter to investigate whether downregulation of SFRP1 was caused by epigenetic hypermethylation of the promoter. We found that the SFRP1 promoter was completely unmethylated ($< 1\%$ methylated) in all three ahNSC cultures, whereas methylation levels ranged from 54% to 99% (Fig. 4C) in the four GSCs cultures. As expected, the degree of methylation was inversely related to *SFRP1* expression (Fig. 4D).

3.4. *Treatment with recombinant SFRP1 reduces GSC proliferation and sphere formation*

We treated three primary GSC cultures with recombinant SFRP1 for 14 days to assess the effect on proliferation and sphere formation. In all three GSC cultures there was a dose-dependent decrease in both proliferation and sphere formation upon treatment with SFRP1 (Fig. 5A). Using the highest concentration (3.2 μg/ml), the reduction in proliferation was 32% for T2609 ($p < 0.001$), 54% for T0836 ($p < 0.000001$), and 51% for T1008 ($p < 0.000001$). The reduction in sphere formation at the highest concentration was 28% for T2609 ($p < 0.01$), 95% for T0836 ($p < 0.000001$) and 96% for T1008 ($p < 0.00001$; Fig. 5A). In addition, we also measured the effect of a shorter, 72-h, SFRP1 treatment. This resulted in a more modest, but significant, reduction in proliferation in two of the three GSC cultures (Supplementary Fig. S2).

The nuclear translocation of β-catenin is a hallmark of Wnt/β-

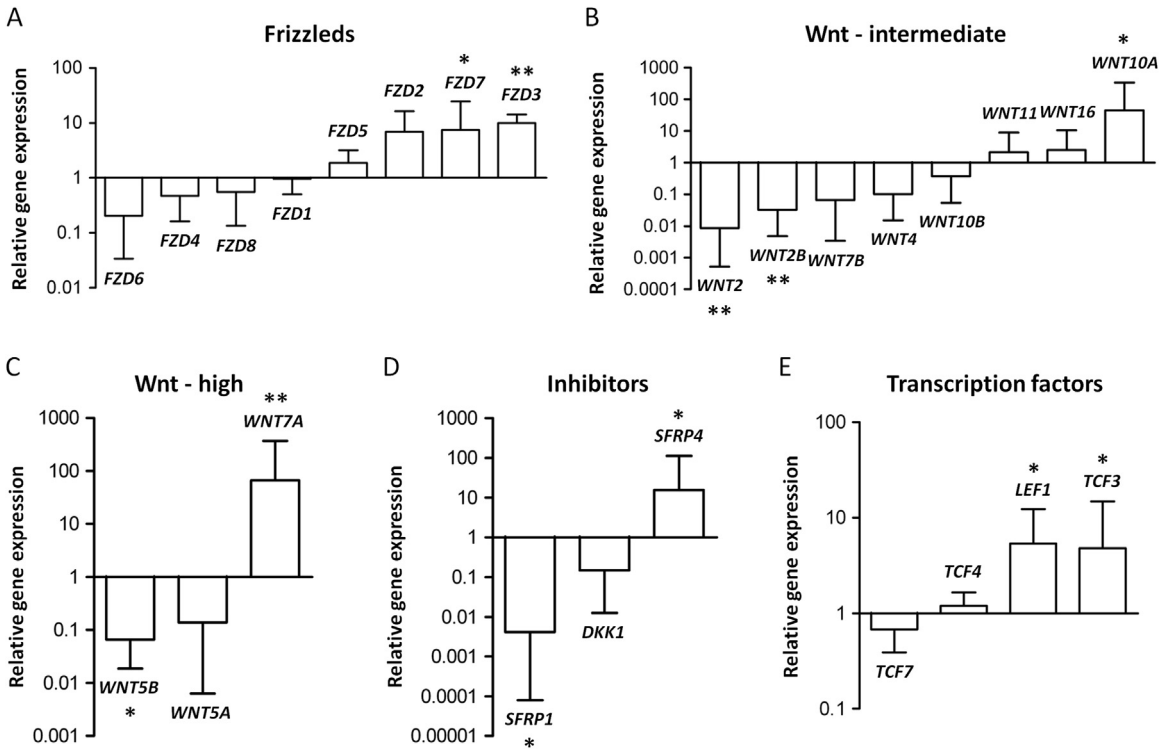

Fig. 2. Expression of Wnt-related genes in GSCs and ahNSCs. (A) Frizzled receptors. (B) Intermediately expressed Wnts. (C) Highly expressed Wnts. (D) Extracellular Wnt inhibitors. (E) Transcription factors. * $p < 0.05$, ** $p < 0.01$.

catenin signaling. To verify that SFRP1 blocks this signaling pathway we performed both immunocytochemistry and western blot of nuclear fractions from cells treated with SFRP1 (3.2 μg/ml) or control medium for 10 days. The immunocytochemistry analysis showed both a reduction in the number of cells expressing active (unphosphorylated) β-catenin (ABC) and an increased expression of GFAP, a differentiation marker (Fig. 5B). This analysis was supplemented with a western blot revealing a reduction of nuclear ABC (Fig. 5C) and an increased expression of GFAP in the SFRP1-treated cells compared to the untreated cells (Supplementary Fig. S3).

In a therapeutic perspective it is important to know whether the effect of SFRP1 on growth is permanent or reversible. For this purpose T0836 was treated with SFRP1 (3.2 μg/ml) or control medium for 10 days, split back to single cells and replated. Proliferation and sphere formation was then measured every seven days for three weeks. At all three time points the SFRP1-treated culture displayed lower cell counts than the control. However, by the end of the third week the SFRP1-treated culture appeared to have reestablished exponential growth (Fig. 5D).

3.5. *SFRP1 downregulates GSC markers and Wnt target genes*

Wnt regulates a variety of cellular processes depending on cell type and context. To investigate the effect of Wnt inhibition in GSCs we compared gene expression in T0836 treated with SFRP1 or control medium for 10 days (3.2 μg/ml) by microarray. At 1% false discovery rate (FDR) the Rank Product algorithm identified 241 upregulated and 145 downregulated genes. Selected genes were confirmed by qPCR (Fig. 6A). We have previously identified genes that are highly expressed in GSCs and that correlate with poor clinical outcome [19]. Treatment with SFRP1 led to the downregulation of three of these genes: *Targeting protein for Xklp2 (TPX2)* was regulated by 0.53 fold, *Kinesin family member 18 A*

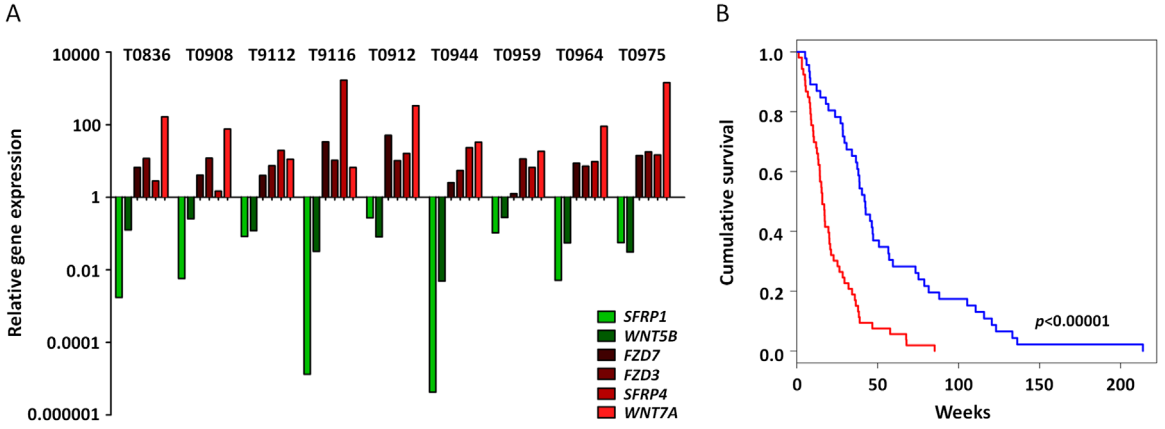

Fig. 3. GSC Wnt signature. (A) Expression of the six genes in nine primary cell cultures. (B) Kaplan–Meier curve calculated for two groups defined by the score from the Cox regression model combining the genes *SFRP1*, *SFRP4* and *FZD7*.

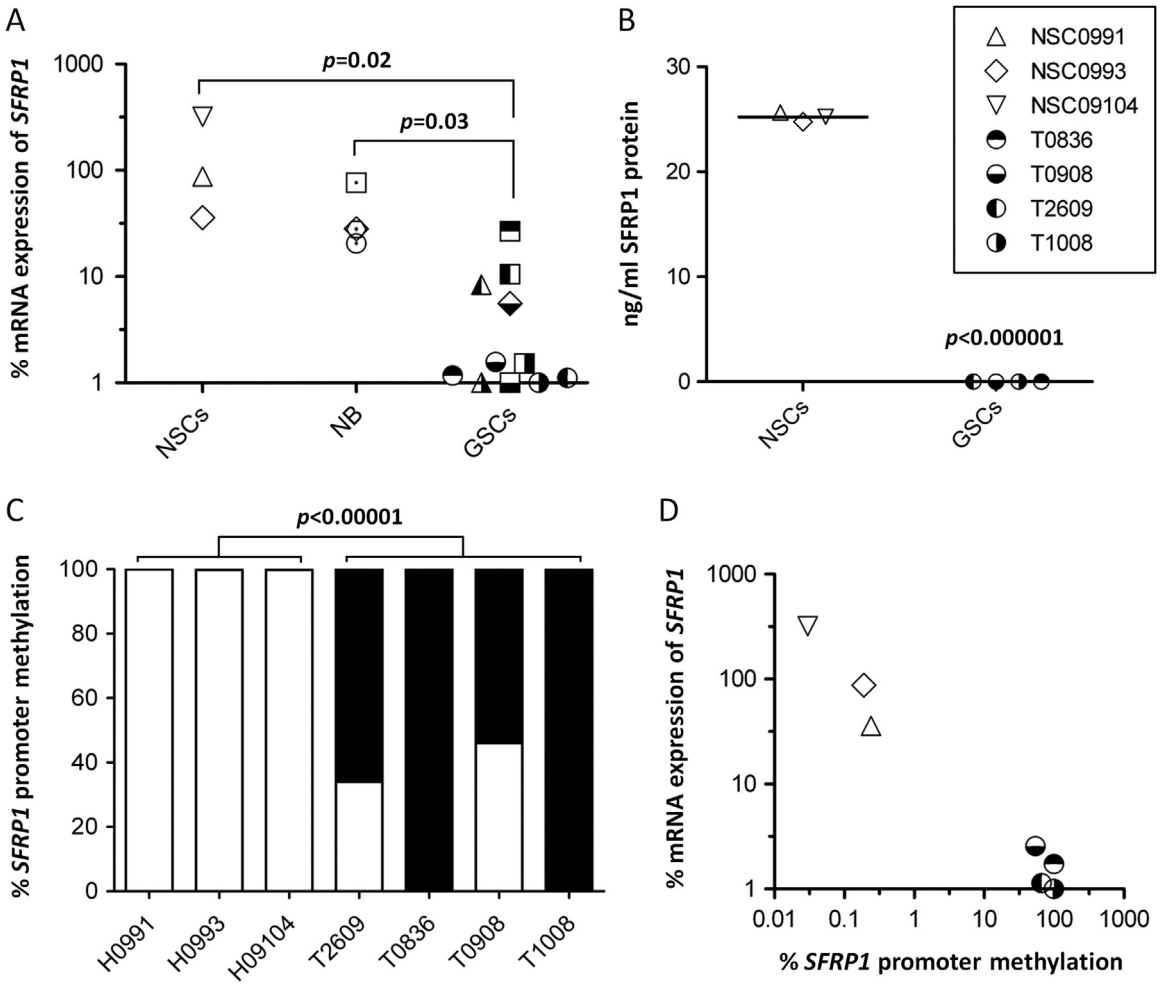

Fig. 4. Downregulation of SFRP1 through epigenetic silencing. (A) mRNA expression of SFRP1 in ahNSCs, normal brain (NB) and GSCs. (B) Concentration of SFRP1 protein detected in culture medium from 5000 cells cultured for seven days (ng/ml). (C) Level of SFRP1 promoter methylation in ahNSCs and GSCs. (D) Correlation between SFRP1 mRNA expression and SFRP1 promoter regulation in ahNSCs and GSCs.

(KIF18A) by 0.76 fold and *DNA topoisomerase 2-alpha (TOP2A)* by 0.74 fold (Fig. 6A). In addition the Wnt target genes *Cyclin D2 (CCDN2)* and *Wnt-inducible signaling protein-1 (WISP1)* were downregulated (0.8 fold and 0.69 fold, respectively), and the Wnt inhibitor *SFRP3* was upregulated by 4.47 fold (Fig. 6A). In addition, the neural differentiation marker *GFAP* was confirmed upregulated by 2.38 fold (Fig. 6A).

3.6. SFRP1 halts cell cycling and induces apoptosis

A signaling pathway enrichment analysis from the list of SFRP1-regulated genes (5% FDR, fold change >2) revealed a significant enrichment of genes related to cell cycle ($p<0.0001$) and p53 signaling ($p<0.0001$; see Supplementary Data). To investigate a potential regulation of the cell cycle by SFRP1 we performed a cell cycle analysis on T0836 treated with SFRP1 or control medium for 10 days. The analysis showed that treatment with SFRP1 reduced the number of cells undergoing cell division from 40% to 12% (Fig. 6C). Furthermore, the fraction of SFRP1-treated cells in G2 phase was reduced from 28% to 7%, combined with an increase in the S-phase fraction from 15% to 26%, suggesting that Wnt inhibition results in a S/G2 transition arrest (data not shown).

p53 is a tumor suppressor involved in cell cycle arrest, as well as induction of apoptosis. To test whether SFRP1 induces apoptosis in GSCs, the same GSC cultures were treated with SFRP1 (3.2 µg/ml) for 48 h. In all three GSC cultures SFRP1 treatment increased apoptosis compared to control, with a significant increase of 54% in T0836 ($p<0.001$) and 42% in T2609 ($p<0.01$; Fig. 6B). To investigate the underlying mechanisms for SFRP1-induced apoptosis, we further investigated protein levels of p53, but did not find any regulation of p53 on the protein level, neither for total protein, nor for the phosphovariants Ser15, Ser20, Ser37 and Ser315 (Supplementary Fig. S3). However, using qPCR we found that two important p53 target genes were upregulated *Growth arrest and DNA-damage-inducible protein 45 alpha* (*GADD45A*) by 2.19 fold, *Cyclin-Dependent Kinase Inhibitor 1 A (CDKN1A*, also called p21) by 2.90 fold, whereas *B-Cell Translocation Gene 2* (*BTG2*) was unchanged, and BAX was downregulated by 0.15 fold (Fig. 6A).

4. Discussion

This study is the first comprehensive investigation of the role of Wnt in CSCs from GBM. By comparing primary GSC- and ahNSC cultures we have identified a Wnt activation signature of six genes involved in Wnt signaling at the cell surface. The combined expression of three of these genes (*FZD7*, *SFRP1* and *SFRP4*) was correlated with clinical outcome in patients with malignant glioma. Furthermore, we demonstrated that treatment with the downregulated inhibitor SFRP1 reduced proliferation and sphere formation through regulation of cell cycle and apoptosis.

This study is based upon *in vitro* exploration of early passage

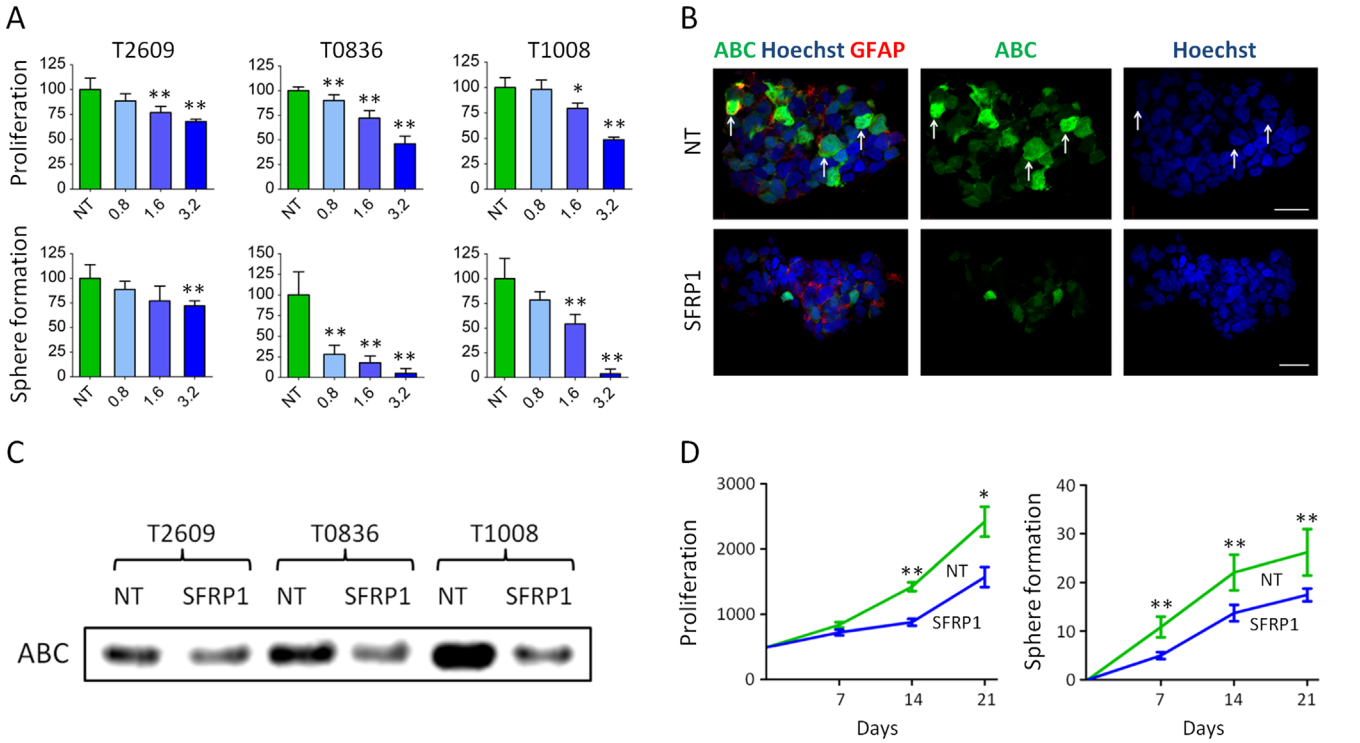

Fig. 5. Treatment with SFRP1 impedes growth through inhibition of Wnt/β-catenin. (A) Proliferation (upper panel) and sphere formation (lower panel) of cells treated with SFRP1 or control medium. Concentrations in μg/ml and results are presented as mean ± SD. (B) Immunocytochemistry of GSC spheres (T1008) showing the expression of active (unphosphorylated) β-catenin (green) and GFAP (red) in non-treated GSCs (upper panel) and SFRP1-treated GSCs (3.2 μg/ml; lower panel). Cell nuclei stained with Hoechst (blue). Scale bar is 20 μm. (C) Western blot of active (unphosphorylated) β-catenin in three primary glioma cultures treated with SFRP1 (3.2 μg/ml) or medium for 10 days. (D) T0836 was treated with SFRP1 (3.2 μg/ml) or medium for 10 days, then replated and cultured for three weeks. The graph shows proliferation and sphere formation of SFRP1-treated cells compared to control after treatment withdrawal. * $p < 0.05$, ** $p < 0.01$.

human derived primary cell cultures. Tumorsphere culturing conditions have been demonstrated to maintain tumorigenicity, genotype and the patient specific characters of individual tumors [6,25,26]. As a result the findings presented in this paper are likely to represent biological mechanisms that are important for at least a subset of GBMs. However, as the work is based on in vitro studies, the importance of Wnt signaling in GBM needs further studies in an *in vivo* setting. Since the stability and availability of SFRP1 is limited, we have not performed such studies currently.

The microarray-based pathway enrichment analysis of signaling pathways revealed a significant regulation of two pathways upon Wnt inhibition: cell cycle and p53 signaling. Through functional analysis we were able demonstrate that SFRP1 halts cell cycling and induces apoptosis. p53 is a tumor suppressor involved in both cell cycle arrest and apoptosis. We did not identify regulation of p53 at the protein level, although two important target genes, *GADD45A* and *CDKN1A* (p21), were upregulated upon SFRP1 treatment.

In addition to regulating a range of embryonic processes, the Wnt signaling pathway is a regulator of somatic stem cells. In hematopoietic stem cells, Wnt is a promoter of self-renewal [27], and in human embryonic stem cells (ESCs) the Wnt receptor FZD7 is essential for maintenance of the undifferentiated, pluripotent state [28]. The Wnt inhibitor SFRP1 may have the opposite effect, promoting ESC differentiation into neural progenitors [29]. Conversely, a reduction in SFRP1 expression enables mammary epithelial cell lines to acquire a $CD44^{high}/CD24^{low}$ expression pattern associated with both mammary and breast cancer stem cells [30], indicating a close relationship between Wnt, stemness and malignancy. Interestingly, *FZD7* and *SFRP1* were among the three genes we found to be correlated with clinical outcome in glioma patients.

This study provides further evidence of the importance Wnt signaling in GBM and GSCs. The expression and nuclear localization of β-catenin has been shown to correlate with glioma grade, and high expression is predictive of poor prognostic outcome in patient tumor biopsies [31–33]. We here present data showing that Wnt plays an important role in GSC proliferation and apoptosis *in vitro*. This is in line with studies performed on cell lines that have shown that Wnt/β-catenin signaling is implicated in oncogenic activities, such as proliferation, inhibition of apoptosis and invasion [20,21]. The upregulation of transcription factors TCF/LEF are also in line with previous findings [20,34].

The Wnt inhibitor SFRP1 is a well-established tumor suppressor in a number of cancers, where it is commonly silenced epigenetically through hypermethylation of the promoter region [35]. Hypermethylation of the *SFRP1* promoter has previously been described in glioma cell tissue [16]. We also found this to be the case in GSCs. Loss of *SFRP1* expression in normal cells has been shown to induce a CSC phenotype [30], and in cancer it is an independent predictor of advanced disease and unfavorable clinical outcome [36–38]. Recent experimental studies indicate that the tumor suppressor effect of SFRP1 includes apoptosis [39] and senescence in [40], which is largely consistent with the findings in this paper.

In contrast to *SFRP1*, *SFRP4* was upregulated in GSCs. Upregulation of *SFRP4* was also associated with an unfavorable clinical prognosis, indicating that SFRP4 may initiate Wnt signaling in GSCs. Such a Wnt-agonistic SFRP4 effect has previously been observed in colorectal cancer cells [41,42]. In fact, SFRP1 and SFRP4 belong to different SFRP subgroups based on sequence homology and antagonize one another's activity [14,43]. Interestingly, a recent study found that SFRP4 increased the chemotherapeutic response in glioma cell lines [44]. More studies are thus needed in order to understand the role of SFRP4 in glioma.

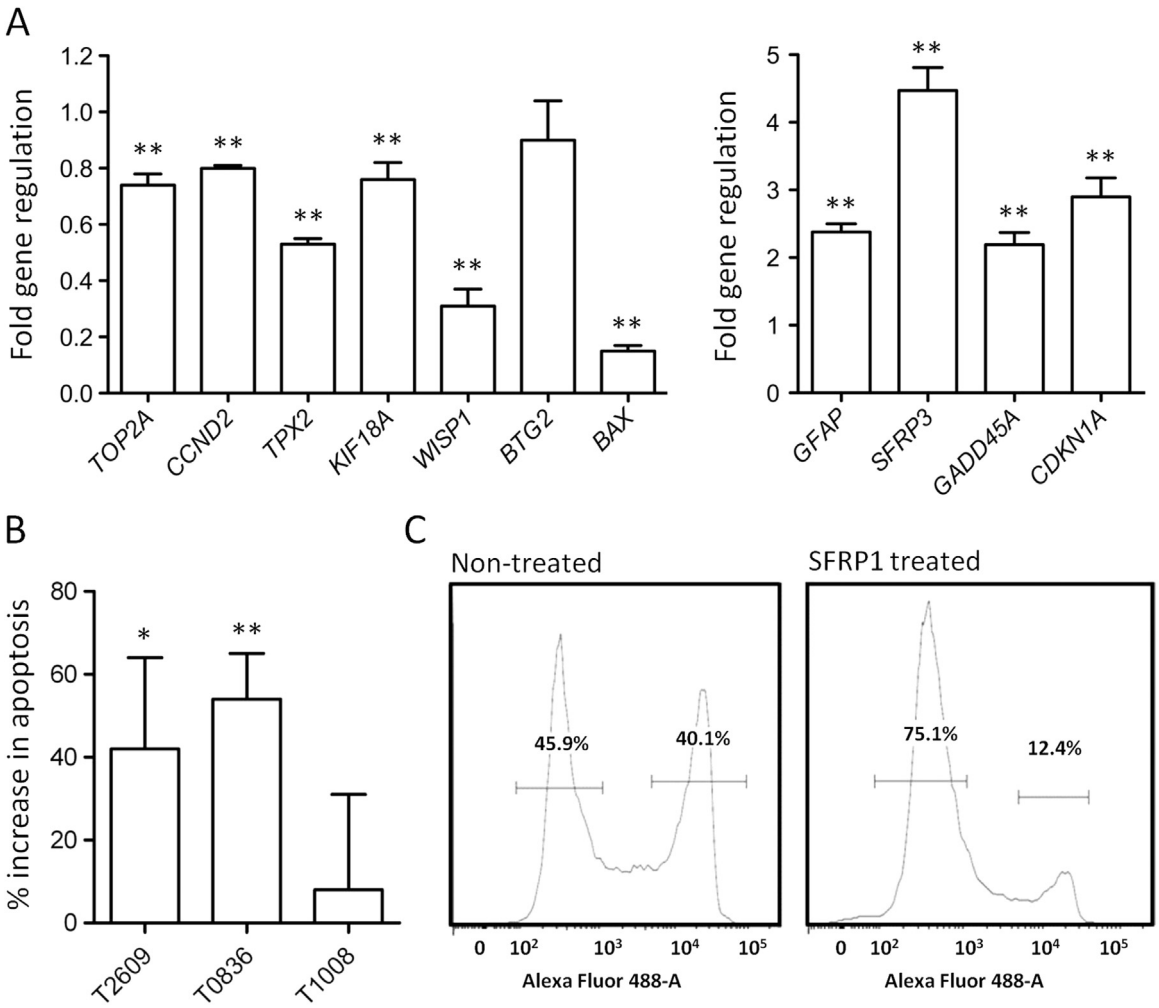

Fig. 6. SFRP1 regulates cell cycle and apoptotic pathways. (A) Microarray analysis of SFRP1-treated cells reveals regulation of cell cycle and p53 pathways. Selected genes were confirmed through qPCR. (B) Measurement of apoptosis in three GSC cultures after 48 h of treatment with SFRP1 of medium. (C) Cell cycle analysis showing a reduction in the number of proliferating cells. * $p < 0.05$, ** $p < 0.01$.

5. Conclusion

Wnt signaling activation through regulation of receptors, ligands and inhibitors appears to be important in GBM stem cell maintenance, and thus tumor propagation. As we have showed that reestablishment of SFRP1 signaling is effective but reversible, further therapeutic strategies should explore the possibilities of non-reversible targeting of this Wnt signaling interaction.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2015.12.010.

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