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Downregulation of SCAI enhances glioma cell invasion and stem cell like phenotype by activating Wnt/β-catenin signaling

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ABSTRACT

SCAI (suppressor of cancer cell invasion), has been recently characterized as a novel tumor suppressor that inhibits the invasive migration of several human tumor cells. However, the expression pattern, biological role and molecular mechanism of SCAI in human glioma remain unknown. In this study, we found that levels of SCAI protein and mRNA expression were significantly downregulated in glioma tissues and cell lines. Overexpression of SCAI inhibited, but silencing of SCAI robustly promoted the invasive and cancer stem cell-like phenotypes of glioma cells. Furthermore, we demonstrated that SCAI downregualtion activated the Wnt/β-catenin signaling, and blockade of the Wnt/β-catenin pathway abrogated the effects of SCAI downregulation on glioma cell aggressiveness. Taken together, our results provide the first demonstration of SCAI downregulation in glioma, and its downregulation contributes to increased glioma cell invasion and self-renewal by activating the Wnt/β-catenin pathway.

1. Introduction

Glioma is one of the most malignant and lethal human cancers with a median survival being only approximately 12–15 months [1]. Recent studies revealed that the robust ability of migration through cerebrum and self renewal are the two most significant glioma phenotypic features, which might contribute to destruction of functional brain tissue, incomplete surgical resection of the tumor mass and extremely high frequency of relapse [2–4]. However, the molecular mechanisms of glioma aggressiveness remains poorly understood, and thus far, no effective prognostic or therapeutic targets have been developed. Thus, delineation of the mechanisms that regulate cell invasion and self-renewal in glioma may allow the identification of novel targets that could serve as possible targets for therapeutic intervention.

The Wnt/β-catenin signaling pathway is constitutive activated in a variety of human cancers, including glioma [5]. The Wnt/β-catenin signaling plays a critical role in tumorigenesis by transcriptional regulation of its downstream genes, such as MYC [6], MMP7 [7], Snail [8] and Sox9 [9], thus leading to cell proliferation, invasion and cancer stem cell property maintenance [10,11]. At this point, targeting the Wnt/β-catenin signaling might be a promising strategy for glioma therapy. Unlike colorectal cancer, in which hyperactivation of β-catenin are frequently found as a result of mutational loss of the adenomatous polyposis coli (APC) gene, or stabilizing mutations in the β-catenin gene itself [12], APC loss or β-catenin gene mutation was rarely found in glioma [13]. Therefore, delineation of the mechanisms that regulate Wnt/β-catenin signaling may provide new clues for the development of targeted cancer therapies for glioma.

SCAI is recently characterized as a putative tumor suppressor that inhibits the invasive migration of human tumor cells through the transcriptional co-repression of MAL/SRF signaling [14,15]. Consistently, reduced SCAI expression was found in several tumors [15]. Notably, SCAI RNA levels are abundant in the brain [14]. However, its expression and role in glioma remains unknown.

In the current study, we found that SCAI was robustly reduced in gliomas tissues and cell lines. Knockdown of SCAI promotes, and ectopic expression of SCAI inhibits the invasive and cancer stem cell-like phenotype of glioma. Furthermore, we demonstrated that SCAI downregulation led to transcriptional activation of the Wnt/β-catenin pathway. Taken together, our results suggest that SCAI act as a tumor suppressor and its downregulation may play an important role in the development and progression of glioma.

2. Materials and methods

2.1. Tissues and cells

Glioma tissues were obtained from the SunYat-Sen Memorial Hospital of Sun Yat-sen University. Normal brain tissues were

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obtained from individuals who died in traffic accidents and confirmed to be free of any pre-existing pathologically detectable conditions. The approval from the Institutional Research Ethics Committee and donators' consents were obtained.

NHA (Normal human astrocyte, Sciencell) were cultured under the condition as the manufacture instructed. Glioma cell lines LN444, SNB19, U251MG, LN18, U118MG, A172 and U87MG, obtained from ATCC, were routinely maintained in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT).

2.2. Plasmids and oligonucleotides

pMSCV/SCAI overexpressing human SCAI was generated by subcloning the PCR-amplified human SCAI coding sequence into pMSCV-puro-retro vector. To silence endogenous SCAI, two RNAi oligonucleotides were cloned into the pSuper-retro-puro vector to generate pSuper-retro-SCAI-RNAi(s), respectively. The shRNA sequences were as following: shRNAi#1: CGGAUGUUACAAGCUC UGGAA; shRNAi#2: CCCAGAUGAAUAAACCAGGAA. Retroviral production was performed in 293T cells. Stable cell lines expressing SCAI or SCAI shRNA(s) were selected for 10 days with 0.5 μg/ml puromycin 48 h after infection. After 10-day selections, the cell lysates prepared from the pooled population of cells in sample buffer were fractionated on SDS–PAGE for the detection of SCAI protein level.

2.3. Western blotting

Cells were harvested in cell lysis buffer (Cell Signaling Technology; Cat#: 9803) and heated for 5 min at 100 °C. Equal quantities of denatured protein samples were resolved on 10% SDS–polyacrylamide gels, and then transferred onto polyvinylidene difluoride membranes (Roche). After blocking with 5% non-fat dry milk in Tris-buffered saline/0.05% Tween 20 (TBST), the membrane was incubated with a specific primary antibody, followed by the horseradixh peroxidase-conjugated secondary antibody. Proteins were visualized using ECL reagents (Pierce). The anti-SCAI and anti-α-Tubulin antibodies were purchased from abcam (Cambridge, MA).

2.4. Real-time PCR

Total mRNA from cultured cells and fresh surgical glioma tissues was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's manual. Real-time PCR was performed using the Applied Biosystems 7900 Sequence Detection system. Expression data were normalized to the mean of housekeeping gene GAPDH to control the variability in expression levels. The primers used are as following: SCAI-up, CTGACTGGCACAGTGGAGAA; SCAI-dn, TCTTTCCCCTTGAGATTGTGA; Oct4-up, GTGGAGGAAGCTGACAACAA; Oct4-dn, GGTTCTCGATACTGGTTCGC; Nanog-up, GATTTGTGGGCCTGAAGAAA; Nanog-dn, ATGGAGGAGGGAAGAGGAGA; Sox2-up, AACCCCAAGATGCACAACT; Sox2-dn, GCTTAGCCTCGTCGATGAAC; CD133-up, CCATTGGCATTCTCTTTGAA; CD133-dn, TTTGGATTCATATGCCTTCTGT; Ascl2-up, GGCACCAACACTTGGAGATT; Ascl2-dn, CCCTCCAGCAGCTCAAGTTA; CCND1-up, TCCTCTCCAAAATGCCAGAG; CCND1-dn, GGCGGATTGGAAATGAACTT; Frizzled 7-up, CGCCTCTGTTCGTCTACCTC; Frizzled 7-dn, GTCGTGTTTCATGATGGTGC; LGR5-up, CAGCGTCTTCACCTCCTACC; LGR5-dn, GTTTCCCGCAAGACGTAACT; MMP7-up, GAGCTACAGTGGGAACAGGC; MMP7-dn, GCATCTCCTTGAGTTTGGCT; Snail-up, CCTTCTCTAGGCCCTGGCT; Snail-dn, AGGTTGGAGCGGTC AGC; Sox9-up, GACGCTGGGCAAGCTCT; Sox9-dn, GTAATCCGGGTGGTCCTTCT; Twist-up, GTCCGCGTCCCACTAGC; Twist-dn, TCCATTTTCTCCTTCTCTGGAA; GAPDH-up, AAGGTGAAGGTCGGAGTCAA; GAPDH-dn, AATGAAGGGGTCATTGATGG.

2.5. Wound healing assay

Cells were seeded on six-well plates with DMEM containing 10% fetal bovine serum (FBS) and grown to confluence. The cells were scratched with a sterile 200-AL pipette tip to create artificial wounds. At 0 and 24 h after wounding, phase-contrast images of the wound healing process were photographed with a 10× objective lens. Eight images per treatment were analyzed to determine averaging position of the migrating cells at the wound edges.

2.6. Transwell matrix penetration assay

Cells (1×10^4) to be tested were plated on the top side of polycarbonate transwell filter (with Matrigel) in the upper chamber of the BioCoat™ Invasion Chambers (BD, Bedford, MA) and incubated at 37 °C for 24 h. Invaded cells on the lower membrane surface were fixed in 1% paraformaldehyde, stained with crystal violet, and counted (Ten random 100×fields per well). Three independent experiments were performed and the data are presented as mean ± standard deviation (SD).

2.7. Luciferase assays

Cells (2×10^4) were seeded in triplicates in 48-well plates and cultured for 24 h. One hundred nanogram of TOP flash or FOP flash luciferase plasmid were transfected into indicated cells using the Lipofectamine 2000 reagent (Invitrogen Co., Carlsbad, CA) according to the manufacturer's recommendation. Luciferase signals were measured 36 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI) according to the manufacturer's protocol. The results were presented as the relative TOP/FOP ratio.

2.8. Chromatin immunoprecipitation (ChIP) assay

Cells (2×10^6) in a 100-mm culture dish were treated with 1% formaldehyde to cross-link proteins to DNA. The cell lysates were sonicated to shear DNA to sizes of 300–1000 bp. Equal aliquots of chromatin supernatants were incubated with 1 μg of an anti-β-catenin, or an anti-IgG antibodies (Millipore, Billerica, MA) overnight at 4 °C with rotation. After reverse cross-link of protein/DNA complexes to free DNA, PCR was performed.

2.9. Statistical analysis

The two-tailed Student's *t*-test was used to evaluate the significance of the differences between two groups of data in all pertinent experiments. A P values <0.05 was considered significant.

3. Results

3.1. SCAI is robustly reduced in gliomas

To investigate the biological role of SCAI in glioma progression, we first examined the expression level of SCAI in gliomas. As shown in Fig. 1A and B, Western blotting analysis revealed that SCAI was differentially downregulated in glioma patient tissues and cell lines, compared with those in normal brain tissues and NHA cells respectively. Meanwhile, using the real-time PCR analysis, we found that the transcript levels of SCAI were also reduced in glioma tissues and cell lines (Fig. 1C and D). Consistently, publicly available microarray data also indicated that expression levels of SCAI transcript were robustly reduced in different glioma subtypes compared to that in non-tumor brain tissues (NCBI/GEO/GSE23400; n = 106, including 23 non-tumor, 26 astrocytomas, 81

glioblastomas and 50 oligodendrogliomas). Taken together, integrated with published data, our results revealed that SCAI was robustly reduced in gliomas.

3.2. Re-constitution of SCAI inhibited glioma cell invasion and cancer stem cell-like phenotype

To investigate the tumor-suppressive role of SCAI in glioma progression, the SNB19 and U87MG cell lines were established to stably overexpress SCAI by retrovirus transfection (Fig. 2A). Consistent with previous reports, our wound healing assay and transwell matrix penetration assay revealed that re-constitution of SCAI significantly repressed the migratory and invasive phenotypes of SNB19 and U87MG cell lines (Fig. 2B and C).

Moreover, the role of SCAI in cancer stem cell-like phenotype was also investigated in our study. As shown in Fig. 2D, overexpression of SCAI robustly inhibited the ability of glioma cells cultured in suspension to generate neuron-sphere. Meanwhile, the neuron-spheres formed by SCAI overexpressing cells were decreased in size and cell number until day 16 (Fig. 2E). Real-time PCR analysis indicated that expression level of the stem cell maintaining factors Oct4, Nanog and Sox2, and cancer stem cell marker CD133 were decreased in SCAI overexpressing SNB19 and U87MG cell lines. Thus, our results suggested that SCAI inhibited the cancer stem cell-like phenotype in glioma.

3.3. Downregulation of SCAI enhances glioma cell invasiveness and cancer stem cell-like phenotype

The effect of SCAI downregulation on glioma progression was further evaluated by knocking down SCAI transcripts in SNB19 and U87MG cell lines by retrovirus SCAI-shRNA(s) infection (Fig. 3A). Strikingly, wound healing and transwell matrix penetration assays indicated that SCAI downregulation dramatically enhanced the migratory and invasive capacities of glioma cells (Fig. 3B, C). Moreover, SCAI silencing markedly increased the self-renewal ability of glioma cells (Fig. 3D). Knocking down SCAI increased the expression of Oct4, Nanog, Sox2 and CD133 (Fig. 3E). Therefore, our results suggested that downregulation of SCAI promoted the aggressive phenotype of glioma cells.

3.4. Downregulation of SCAI activated Wnt/β-catenin signaling

It has been demonstrated that Wnt/β-catenin signaling plays an important role in promoting glioma cell invasion and stem cell-like phenotype [16,17]. As expected, the β-catenin/TCF activity was significantly repressed by SCAI overexpression, but enhanced by SCAI silencing (Fig. 4A and B). Real-time PCR analysis revealed that the expression levels of eight classically recognized Wnt/β-catenin target genes were decreased in the SCAI overexpressing glioma cells but increased in the SCAI silencing cells (Fig. 4C). Furthermore, using the chromatin immunoprecipitation (CHIP) assay, we found that the recruitment of β-catenin to the promoters of its downstream genes *MMP7*, *Snail* and *Sox9* was inhibited by SCAI expression (Fig. 4D). Taken together, our results suggested that overexpression of SCAI inhibited, but downregulation of SCAI activated Wnt/β-catenin signaling.

3.5. Blockade of the Wnt/β-catenin pathway abrogated SCAI downregulation mediated glioma cell aggressiveness

Moreover, we found that inhibition of Wnt/β-catenin signaling, through TCF dominant-negative mutant (TCF-dn) transfection, dramatically decreased the invasive and self-renewal abilities of SCAI silencing SNB19 and U87MG cells (Fig. 4E and F), suggesting that activation of Wnt/β-catenin pathway is functionally relevant to SCAI downregulation mediated glioma cell aggressiveness.

4. Discussion

Due to the invasive nature and stem cell-like properties of glioma cells, above 90% cases of recurrent gliomas instantaneously develop within several centimeters of the resected region or even adjoining to resected margin [18,19]. Thus, better understanding of the mechanisms underlying glioma infiltration is always one of the hottest spot in glioma research. Herein, we identify a novel

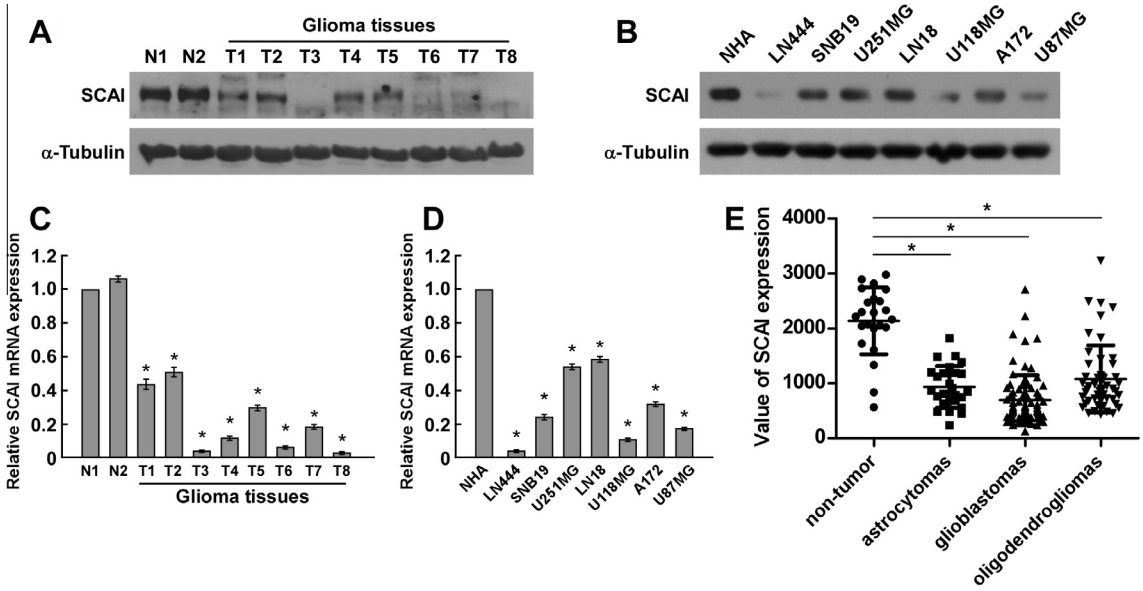

Fig. 1. Protein and mRNA expression levels of SCAI is differentially downregulated in human glioma tissues and cell lines. (A and B) Western blotting analysis of SCAI protein expression in 2 normal brain tissues and 8 human glioma tissues (A), and in NHA and 7 glioma cell lines (B). α-Tubulin served as the loading control. (C and D) Real-time PCR analysis of SCAI mRNA expression in corresponding glioma tissues and cells. Transcript levels were normalized by GAPDH expression. (E) Expression levels of SCAI transcript was robustly reduced in different glioma subtypes compared to that in non-tumor brain tissues (NCBI/GEO/GSE23400; $n = 106$, including 23 non-tumor, 26 astrocytomas, 81 glioblastomas and 50 oligodendrogliomas). Each bar represents the mean of three independent experiments. $^*P < 0.05$.

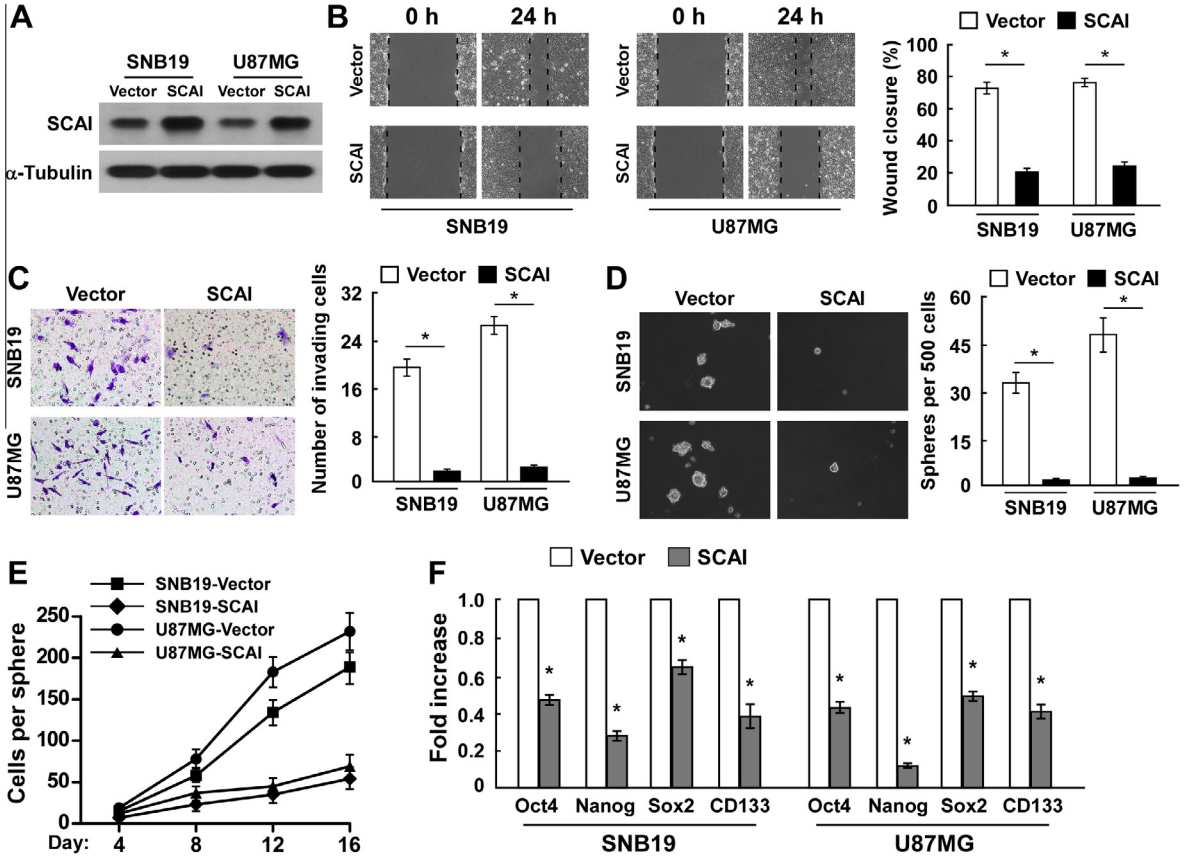

Fig. 2. Re-constitution of SCAI inhibited glioma cell invasion and cancer stem cell-like phenotype. (A) Western blotting analysis of SCAI expression in SNB19 and U87MG cells infected with SCAI-ORF or vector control. α-Tubulin served as the loading control. (B) Migration of indicated cells analyzed by the wound healing assay. (C) Representative micrographs (left) and quantification (right) of invading cells were analyzed using the transwell matrix penetration assay. (D) Representative images (left) and quantification (right) of neuron spheres formed by the indicated cells. (E) The number of cells per sphere in spheres formed by the indicated cells at indicated time. (F) Real-time analysis of Oct4, Nanog, Sox2 and CD133 transcript level in indicated cells. Transcript levels were normalized by GAPDH expression. Each bar represents the mean of three independent experiments. $^{*}P < 0.05$.

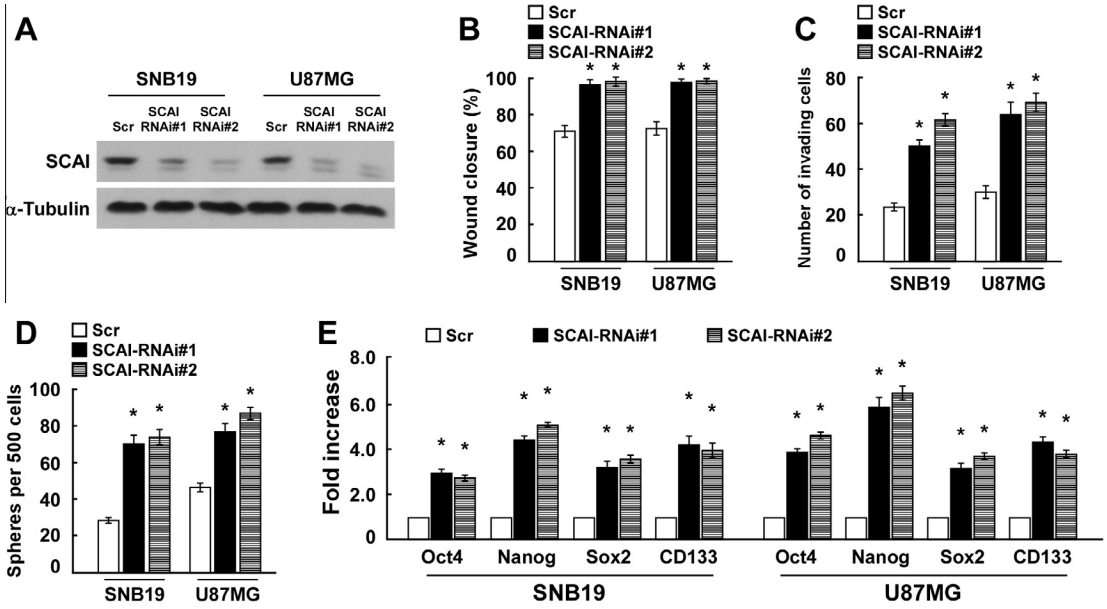

Fig. 3. Downregulation of SCAI enhances glioma cell invasiveness and cancer stem cell-like phenotype. (A) Western blotting analysis of SCAI expression in SNB19 and U87MG cells infected with SCAI-RNAi#1, SCAI-RNAi#2 or scramble control. α-Tubulin served as the loading control. (B) Migration of indicated cells analyzed by the wound healing assay. (C) Quantification of invaded cells were analyzed using the transwell matrix penetration assay. (D) Quantification of neuron spheres formed by the indicated cells. (E) Real-time PCR analysis of Oct4, Nanog, Sox2 and CD133 expression in SCAI-RNAi(s) cells compared to scramble control cells. Transcript levels were normalized by GAPDH expression. Each bar represents the mean of three independent experiments. $^{*}P < 0.05$.

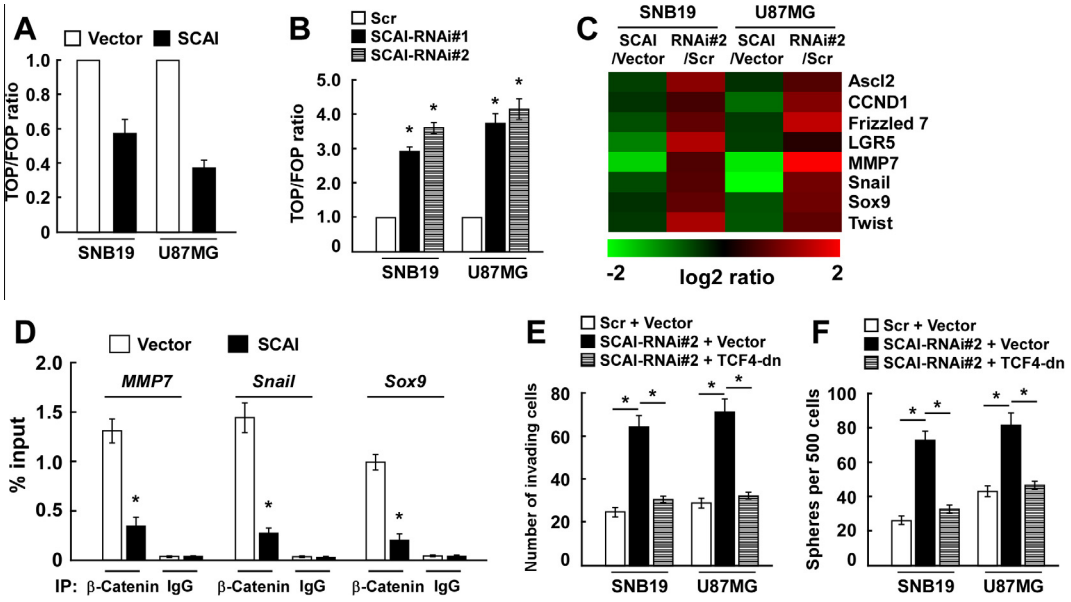

Fig. 4. Downregulation of SCAI activated Wnt/β-catenin signaling. (A and B) TOP/FOP luciferase ratio reported Wnt/β-catenin pathway activity in SCAI overexpressing cells compared to control cells (A), and SCAI silencing cells compared to control cells (B). (C) Real-time PCR analysis indicating an apparent overlap between Wnt/β-catenin-dependent gene expression and SCAI-regulated gene expression. The pseudocolor represents the intensity scale of SCAI versus vector, or SCAI shRNA#2 versus scramble, generated by a log2 transformation. (D) ChIP assays were performed in SCAI-overexpressing and control cells using an anti-β-catenin antibody to identify β-catenin recruitment in the *MMP7*, *Snail* and *Sox9* promoter. IgG was used as a negative control. (E and F) Blockade of the Wnt/β-catenin pathway via overexpressing TCF4-dn inhibits SCAI downregulation-induced glioma cell invasion (E) and self-renewal (F).

mechanism mediated by the downregulation of SCAI. Specifically, we found that downregulation of SCAI induced, while re-constitution of SCAI highly inhibited glioma cell invasion and self-renewal. Furthermore, we demonstrated that downregulation of SCAI activated the Wnt/β-catenin signaling pathway, leading to production of its downstream genes such as MMP7, Sox9 and Snail, which are responsible for SCAI biological functions. Therefore, our results may not only suggest a new mechanism underlying glioma aggressiveness, but provide new clues for therapeutic intervention.

Constitutive activation of Wnt/β-catenin signaling pathway is common in a wide range of human solid tumors and plays a central role in tumorigenesis by typing the balance towards cell proliferation, invasion, metastasis and stem cell-like properties [10,11,20]. Since the frequency of APC loss or β-catenin mutation is low in glioma [13], our findings thus present a novel mechanism by which SCAI downregulation induced Wnt/β-catenin signaling activation in cancer. Considering that SCAI functions as a transcriptional co-repressor, it might be of great interest to investigate whether SCAI inhibit Wnt/β-catenin signaling by direct interacting with β-catenin or TCF4 protein in the future.

Previously, only the suppressive role of SCAI in tumor cell invasion was identified [14,15]. Notably, here we demonstrated that SCAI also inhibited the self-renewal ability of tumor cells, suggesting that SCAI might function as a pleiotropic modulator suppressing cancer progression. Therefore, the biological roles of SCAI in tumor progression demand further exploration.

In summary, our studies first characterized the tumor suppressor role of SCAI in glioma. SCAI was downregulated in glioma tissues and cell lines, contributing to the invasive and cancer stem cell-like phenotypes of glioma cells by activating Wnt/β-catenin signaling. Considering that the research about SCAI is just at the start, the functions and molecular mechanisms for its tumor suppressor features remain to be explored.

Competing interests

The authors declare no competing financial interests.

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