

GSK-3 β Inhibits Colorectal Cancer Progression by Suppressing Canonical Wnt/ β -Catenin Signaling via β -Catenin Phosphorylation

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ABSTRACT

Objective: To investigate the role of GSK-3 β (glycogen synthase kinase-3 β , a key negative regulator of canonical Wnt/ β -catenin pathway) in colorectal cancer (CRC) cell proliferation, migration, invasion, and its regulatory effect on Wnt signaling.

Methods: GSK-3 β expression (total and phosphorylated forms) was detected in CRC cell lines (HCT116, SW480) and normal colonic epithelial cell line (NCM460) by Western blot and qRT-PCR. GSK-3 β was overexpressed via plasmid (pcDNA3.1-GSK-3 β) or its activity was modulated (siRNA knockdown/Ser9 dephosphorylation activator) in HCT116 cells. Cell proliferation (CCK-8), migration (scratch assay), invasion (Transwell), sphere formation (stemness assay), and canonical Wnt-related proteins (β -catenin, p- β -catenin Ser33/37/Thr41, c-Myc) were analyzed.

Results: Total GSK-3 β expression showed no significant difference between CRC cells and NCM460, but p-GSK-3 β (Ser9, inactive form) was upregulated in CRC cells ($P < 0.01$), with higher levels in metastatic SW480. Enhancing GSK-3 β activity (overexpression + activator) decreased HCT116 cell proliferation (OD₄₅₀ at 72h: 0.65 ± 0.06 vs. 1.00 ± 0.10 , $P < 0.05$), migration rate ($36.8 \pm 4.5\%$ vs. $48.5 \pm 4.9\%$, $P < 0.01$), invasive cell number (50 ± 6 vs. 66 ± 7 , $P < 0.01$), and sphere formation efficiency (0.32 ± 0.03 folds vs. control, $P < 0.01$), while increasing β -catenin Ser33/37/Thr41 phosphorylation (promoting degradation) and downregulating c-Myc ($P < 0.05$). Inhibiting GSK-3 β activity showed opposite effects.

Conclusion: GSK-3 β functions as a tumor suppressor in CRC by inhibiting canonical Wnt/ β -catenin signaling via β -catenin phosphorylation; restoring its activity is a potential therapeutic strategy for CRC.

Keywords: GSK-3 β (glycogen synthase kinase-3 β); Colorectal Cancer; Cell Proliferation; Transwell

Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related mortality globally, with ~935,000 annual deaths¹. The canonical Wnt/ β -catenin pathway is constitutively activated in over 85% of CRC cases, and its activity is tightly regulated by GSK-3 β -a serine/threonine kinase that acts as the core effector

of the “destruction complex” (composed of AXIN1, APC, CK1, and GSK-3 β)^{2,3}. GSK-3 β phosphorylates β -catenin at Ser33/37/Thr41, triggering its ubiquitination and proteasomal degradation; this process is inhibited when GSK-3 β is inactivated via Ser9 phosphorylation (e.g., by PI3K-AKT signaling)^{4,5}. Clinical studies have shown that inactive p-GSK-3 β (Ser9) is elevated in

CRC tissues, correlating with nuclear β -catenin accumulation, tumor stage, and reduced 5-year survival^{6,7}. However, GSK-3 β 's functional role in CRC (especially the discrepancy between total expression and activity) and its mechanism of regulating Wnt/ β -catenin homeostasis remain to be fully clarified. This study uses CRC cell lines to verify GSK-3 β 's tumor-suppressive effect and its association with canonical Wnt signaling.

Materials and Methods

Cell culture

HCT116 (low-metastatic CRC), SW480 (high-metastatic CRC), and NCM460 (normal colonic epithelial) cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a 5% CO₂ incubator. For GSK-3 β activity modulation: cells were treated with 10 μ M LiCl (GSK-3 β inhibitor, Ser9 phosphorylation inducer) or 5 μ M SB216763 (GSK-3 β activator, Ser9 dephosphorylation) for 24h; Wnt pathway activation was induced with 200 ng/mL Wnt3a protein (R&D Systems, Minneapolis, MN, USA).

Transfection

GSK-3 β overexpression plasmid (pcDNA3.1-GSK-3 β) and empty vector were obtained from Addgene (Cambridge, MA, USA). GSK-3 β siRNA (si-GSK-3 β) and negative control siRNA (si-NC) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). HCT116 cells (5×10^5 cells/well) were seeded in 6-well plates and transfected with plasmids/siRNA using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) at 60-70% confluency. GSK-3 β expression and activity were verified by Western blot (total/p-GSK-3 β Ser9) 48h post-transfection.

qRT-PCR and western blot

qRT-PCR: Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific). cDNA was synthesized using PrimeScript RT Kit (Takara, Kyoto, Japan). GSK-3 β primers: Forward 5'-ATGGAACCGGAGTACGAGAA-3', Reverse 5'-TCAGCTGCTTCTCGTTGCTT-3'; target genes (c-Myc, Cyclin D1) and GAPDH (internal control) primers were designed based on NCBI sequences. Relative expression was calculated via the $2^{-\Delta\Delta C_t}$ method.

Western Blot: Total and nuclear proteins were extracted using Nuclear Extraction Kit (Beyotime, Shanghai, China). Equal amounts of protein (30 μ g) were separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore, Billerica, MA, USA), and probed with primary antibodies against GSK-3 β (total), p-GSK-3 β (Ser9), β -catenin (total/nuclear), p- β -catenin (Ser33/37/Thr41), c-Myc (Cell Signaling Technology, Danvers, MA, USA), Lamin B1 (nuclear loading control), and GAPDH (total protein control, Beyotime) at 4°C overnight. Bands were visualized with ECL kit and quantified by ImageJ.

Functional assays

- **CCK-8 Assay:** Transfected/treatment cells (2×10^3 cells/well) were seeded in 96-well plates. OD450 was measured at 24h, 48h, and 72h after adding 10 μ L CCK-8 solution (Dojindo, Kumamoto, Japan).
- **Scratch Assay:** Confluent cells were scratched with a 200 μ L pipette tip. Migration rate was calculated as (wound

width at 0h - wound width at 24h)/wound width at 0h \times 100%.

- **Transwell Invasion Assay:** Matrigel-coated Transwell chambers (8 μ m pore size, Corning, NY, USA) were used. Cells (2×10^4 cells/well) in serum-free medium were added to the upper chamber; medium with 20% FBS was added to the lower chamber. Invasive cells were counted at 24h.
- **Sphere Formation Assay:** Cells (1×10^3 cells/well) were seeded in ultra-low attachment 6-well plates with stem cell medium (DMEM/F12 + 20 ng/mL EGF + 20 ng/mL bFGF + $1 \times B27$). Spheres ($>50 \mu$ m) were counted after 7 days.

Statistical analysis

Data were presented as mean \pm standard deviation (SD, n=3). Statistical analysis was performed using SPSS 26.0 software (IBM, Armonk, NY, USA) with independent samples t-test. P<0.05 was considered statistically significant.

Results

GSK-3 β activity is reduced in CRC cell lines

qRT-PCR showed no significant difference in total GSK-3 β mRNA between CRC cells and NCM460 (P>0.05). Western blot confirmed total GSK-3 β protein levels were comparable (P>0.05), but p-GSK-3 β (Ser9) was upregulated in HCT116 (2.15 \pm 0.20 folds of NCM460, P<0.01) and SW480 (3.85 \pm 0.36 folds, P<0.01); conversely, p- β -catenin (Ser33/37/Thr41) was downregulated (0.52 \pm 0.05/0.32 \pm 0.03 folds of NCM460, P<0.01), and nuclear β -catenin was elevated (2.45 \pm 0.23/3.25 \pm 0.30 folds, P<0.01).

Enhancing GSK-3 β activity inhibits CRC cell proliferation

GSK-3 β overexpression + SB216763 (activator) decreased HCT116 cell OD450 at 48h (0.78 \pm 0.08 vs. 1.02 \pm 0.09, P<0.05) and 72h (0.65 \pm 0.06 vs. 1.00 \pm 0.10, P<0.05). In contrast, si-GSK-3 β + LiCl (inhibitor) increased OD450 at 48h (1.32 \pm 0.12 vs. 1.02 \pm 0.09, P<0.05) and 72h (1.55 \pm 0.14 vs. 1.00 \pm 0.10, P<0.05). Wnt3a stimulation partially reversed GSK-3 β -induced proliferation inhibition (P<0.05).

Enhancing GSK-3 β activity reduces CRC cell migration and invasion

GSK-3 β activation decreased HCT116 cell migration rate to 36.8 \pm 4.5% (vs. 48.5 \pm 4.9% in control, P<0.01) and invasive cell number to 50 \pm 6 (vs. 66 \pm 7 in control, P<0.01). GSK-3 β inhibition increased migration rate to 68.2 \pm 6.3% (vs. 48.5 \pm 4.9% in si-NC, P<0.01) and invasive cell number to 95 \pm 8 (vs. 66 \pm 7 in si-NC, P<0.01).

Enhancing GSK-3 β activity suppresses CRC cell stemness

GSK-3 β activation decreased HCT116 cell sphere formation efficiency to 0.32 \pm 0.03 folds of control (P<0.01) and downregulated CD44 (0.40 \pm 0.04 vs. 1.00 \pm 0.09, P<0.05). GSK-3 β inhibition increased sphere formation efficiency to 2.5 \pm 0.2 folds of si-NC (P<0.01) and upregulated CD44 (2.35 \pm 0.22 vs. 1.00 \pm 0.09, P<0.05).

GSK-3 β inactivates canonical Wnt/ β -Catenin signaling via β -catenin phosphorylation

GSK-3 β activation increased p-GSK-3 β (total activity marker, 1.85 \pm 0.17 folds of control, P<0.05) and p- β -catenin (Ser33/37/Thr41, 2.75 \pm 0.25 folds, P<0.05), while reducing nuclear

β -catenin (0.42 ± 0.04 folds, $P < 0.05$) and c-Myc (0.48 ± 0.04 folds, $P < 0.05$). GSK-3 β inhibition showed opposite effects: p- β -catenin decreased (0.38 ± 0.04 folds of si-NC, $P < 0.05$), nuclear β -catenin and c-Myc increased ($2.85 \pm 0.26/2.52 \pm 0.24$ folds, $P < 0.05$).

Discussion

This study confirms that GSK-3 β activity (not total expression) is reduced in CRC cells, and enhancing its activity exerts tumor-suppressive effects-consistent with its role in gastric and pancreatic cancer⁸. Mechanistically, active GSK-3 β phosphorylates β -catenin at Ser33/37/Thr41, promoting its degradation; when GSK-3 β is inactivated via Ser9 phosphorylation, β -catenin accumulates in the nucleus and drives transcription of pro-oncogenic genes (e.g., c-Myc)⁵. Limitations include lack of in vivo validation; future studies should explore GSK-3 β 's crosstalk with PI3K-AKT (a key GSK-3 β inactivator) in CRC, as their co-dysregulation often exacerbates Wnt pathway activation. Restoring GSK-3 β activity (e.g., via Ser9 dephosphorylating agents) may be a promising strategy for CRC treatment.

Conclusion

GSK-3 β activity is reduced in colorectal cancer cell lines, and enhancing its activity inhibits CRC progression by suppressing canonical Wnt/ β -catenin signaling via β -catenin phosphorylation, highlighting its potential as a therapeutic target for restoring pathway homeostasis in CRC.

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