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Research Article

GSK-3β 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-3β (glycogen synthase kinase-3β, 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-3β 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-3β was overexpressed via plasmid (pcDNA3.1-GSK-3β) 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-3β expression showed no significant difference between CRC cells and NCM460, but p-GSK-3β (Ser9, inactive form) was upregulated in CRC cells ($P<0.01$), with higher levels in metastatic SW480. Enhancing GSK-3β 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±4.5% vs. 48.5±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-3β activity showed opposite effects.

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

Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related mortality globally, with ~935,000 annual deaths[1]. The canonical Wnt/β-catenin pathway is constitutively activated in over 85% of CRC cases, and its activity is tightly regulated by GSK-3β-a serine/threonine kinase that acts as the core effector of the "destruction complex" (composed of AXIN1, APC, CK1, and GSK-3β)[2,3]. GSK-3β phosphorylates β-catenin at Ser33/37/Thr41, triggering its ubiquitination and proteasomal degradation; this process is inhibited when GSK-3β is inactivated via Ser9 phosphorylation (e.g., by PI3K-AKT signaling)[4,5]. Clinical studies have shown that inactive p-GSK-3β (Ser9) is elevated in

CRC tissues, correlating with nuclear β-catenin accumulation, tumor stage, and reduced 5-year survival[6,7]. However, GSK-3β'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-3β'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% CO_2 incubator. For GSK-3β activity modulation: cells were treated with 10 μM LiCl (GSK-3β inhibitor, Ser9 phosphorylation inducer) or 5 μM SB216763 (GSK-3β activator, Ser9 dephosphorylation) for 24h; Wnt pathway activation was induced with 200 ng/mL Wnt3a protein (R&D Systems, Minneapolis, MN, USA).

Transfection

GSK-3β overexpression plasmid (pcDNA3.1-GSK-3β) and empty vector were obtained from Addgene (Cambridge, MA, USA). GSK-3β siRNA (si-GSK-3β) 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-3β expression and activity were verified by Western blot (total/p-GSK-3β 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-3β 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 $2^{-}\Delta\Delta Ct$ method.

Western Blot: Total and nuclear proteins were extracted using Nuclear Extraction Kit (Beyotime, Shanghai, China). Equal amounts of protein (30μg) were separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore, Billerica, MA, USA), and probed with primary antibodies against GSK-3β (total), p-GSK-3β (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 10μL CCK-8 solution (Dojindo, Kumamoto, Japan).
- **Scratch Assay:** Confluent cells were scratched with a 200μL pipette tip. Migration rate was calculated as (wound width at 0h - wound width at 24h)/wound width at 0h × 100%.
- **Transwell Invasion Assay:** Matrigel-coated Transwell chambers (8μm 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× B27). Spheres (>50 μm) were counted after 7 days.

Statistical analysis

Data were presented as mean ± 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-3β activity is reduced in CRC cell lines

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

Enhancing GSK-3β activity inhibits CRC cell proliferation

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

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

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

Enhancing GSK-3β activity suppresses CRC cell stemness

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

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

GSK-3β activation increased p-GSK-3β (total activity marker, 1.85±0.17 folds of control, $P<0.05$) and p-β-catenin (Ser33/37/Thr41, 2.75±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-3β 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±0.26/2.52±0.24 folds, P<0.05).

Discussion

This study confirms that GSK-3β 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 cancer[8]. Mechanistically, active GSK-3β phosphorylates β-catenin at Ser33/37/Thr41, promoting its degradation; when GSK-3β 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-3β's crosstalk with PI3K-AKT (a key GSK-3β inactivator) in CRC, as their co-dysregulation often exacerbates Wnt pathway activation. Restoring GSK-3β activity (e.g., via Ser9 dephosphorylating agents) may be a promising strategy for CRC treatment.

Conclusion

GSK-3β 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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