Molecular characterization of Axin2-GSK3β binding site

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Molecular characterization of Axin2-GSK3β binding site

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ABSTRACT

Molecular characterization of Axin2-GSK3β binding sites

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About 90% of human cancers originate from epithelial tissue, and epithelial mesechymal transition (EMT) is related in the conversion of early stage tumors into invasive malignancies. Wnt signaling inhibits Snail phosphorylation through Axin2-dependent nuclear export of GSK3β, then consequently increases E-cadherin repressor, Snail protein levels and induces an EMT. The important roles of LRP5/6 on Axin activation had been described, but it's roles of nuclear GSK3β compartmentalization had not been well-known. According to the structure of Axin-GSK3β complex reported recently, the Axin makes several hydrophobic interactions and only a single hydrogen bond to GSK3β. Hydrophobic amino acid residues of Axin form a helical 'ridge' that packs into a hydrophobic 'groove' formed between helix and the extended loop in GSK3β. The possibility of molecular target for Axin2-GSK3β binding sites had not been verified yet. Thus, this study was aimed to elucidate the

regulatory cascade of GSK3β compartmentalization by Wnt co-receptor, LRP5/6 and clarify the roles of Axin2 during the process, and to verify potential therapeutic target for Axin2-GSK3β complex though validate the anti-EMT effect of chemical candidates blocking the principal Axin2-GSK3β protein-protein interaction sites.

The results are as follows:

1. LRP6 stabilizes Snail and sustains its nuclear accumulation by Axin2-dependent nuclear export of GSK3β.

2. Amino acid residues of Y216 and V267/268 in GSK3β is crucial binding sites to interact with Axin2 according to *in vitro* binding assay with GSK3β mutants.

3. Hydrophobic amino acid residues of Axin2, L374 and L378 form principal Axin2-GSK3β protein-protein interaction, but a single hydrogen bonding residue R377 is not critical for Axin2-GSK3β binding.

4. The target molecule of Axin2-GSKβ interaction, selected by pharmacophore model, increases nuclear GSK3β expression and its activity, consequently decreases Snail expression. The target also increases E-cadherin expression, and inhibits cancer cell migration.

The results of the study clarified the functional regulation of GSK3β nucleocytoplasmic compartmentalization mediated by LRP5/6 and Axin2, and verified the possibility of molecular target for Axin2-GSK3β binding sites as

anti-EMT target.

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Key Words : LRP5/6, Axin2, GSK3β, Nuclear export function, Wnt signaling pathway, Epithelial-mesenchymal transition (EMT)

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I. INTRODUCTION

Epithelial cells can acquire plasticity and migratory activity by the process termed epithelial-mesechymal transition (EMT), a complex program wherein epithelial cells display loosening cell-cell contacts and adopt a fibroblastic phenotype. Down-regulation of E-cadherin is a hallmark of EMT, which is induced by Snail via its binding to the E-cadherin promoter region[1]. Indeed, Snail plays a required role in driving the EMT programs during development that mark gastrulation as well as neural crest development[1-5]. In a similar, but misdirected fashion, neoplastic cells co-opt Snail function to adopt a mesenchymal cell-like invasive phenotype that characterizes their aberrant behavior[1-6].

Wnt signaling is highly conserved throughout the evolution, and essential for

animal development and is commonly involved in human diseases, with inappropriate activation of Wnt signaling contributes to mammalian tumorigenesis[7-9]. The transmission of Wnt signals occurs when Wnt acts on target cells in a paracrine manner. In the absence of the Wnt signal, GSK-3β β-catenin is phosphorylated by destruction complex that includes Axin, serine/threonine kinase glycogen synthase-3β (GSK-3β), and adenomatous polyposis coli (APC). The phosphorylated β-catenin is recognized by the mammalian homologue of Slimb, β-TrCP, which is a component of an E3 ubiquitin ligase. β-TrCP associates with the β-catenin and activates the ubiquination machinery resulting in its degradation in proteasomes[10]. Recent study demonstrated the transcriptional repressor of E-cadherin, Snail has a series of β-catenin-like phosphorylation motifs, which imply that Snail is GSK3β-dependent phosphorylation, β-TrCP-directed ubiquitination, and proteosomal degradation[11]. Hence, in normal status, both β-catenin and Snail are repressed and E-cadherin-mediated cell-cell adhesion is preserved (Fig 1. left panel). Secreted Wnt bind to seven-transmembrane Frizzled receptor (Fz) and LDL Receptor-Related Proteins (LRPs) co-receptor, LRP5 or LRP6 on the cell surface and transduces the signal to binding cytoplasmic proteins, dishevelled protein (Dvl) and Axin. It has been already demonstrated that Dvl inhibits GSK3β activity, but the mechanism is not clarified yet. Recent studies revealed that Axin2 regulate nucleocytoplasmic trafficking of GSK3β and induces Snail-mediated EMT[12,13]. These signals lead to functional inactivation and dissociation of a multi-protein destruction complex, and resulted in dephosphorylation and dissociation of both β-catenin and Snail. Unphosphorylated β-catenin and Snail are stabilized and accumulated in the

nucleus of cells. The β-catenin then leads to make a complex with the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of transcription factors in the nucleus leading to the eventual transcription and expression of target genes[10,14,15]. In tandem way, Snail mediates transctional E-cadherin repression and control an EMT (Figure 1, right panel). The membrane anchored form of the intracellular domain LRP5/6 facilitated the recruitment of Axin to the membrane[16,17]. The important roles of LRP5/6 on Axin activation had been described, but it's roles of nuclear GSK3β compartmentalization had not been well-known.

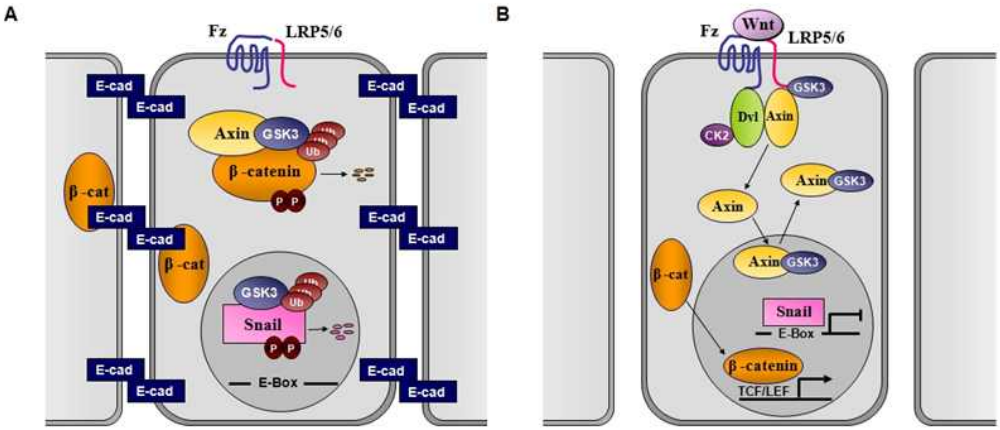

Fig. 1. Outline of Wnt/β-catenin signaling.

(A) In the absence of Wnt, cytosolic β-catenin and Snail are phosphorylated by GSK3, then ubiquitinated and degraded. E-cadherin-mediated cell adhesion is maintained.

(B) Upon Wnt binding to Fz-LRP receptors, a combination of LRP-Axin interaction and Dvl phosphorylation (P) blocks the APC-Axin-GSK3 complex

from phosphorylating β-catenin. Wnt signaling also activates Axin-dependent nuclear export of GSK3, and then nuclear levels of Snail as well as β-catenin are increased, resulting in disruption of cell adhesion and induction of the invasive program.

Recently, the crystal structure of Axin1-GSK3β has been reported. The crystal structure of pTyr216-GSK3β binds to a 19 residue, minimal GSK3β -binding peptide derived from Axin1[18]. The Axin-derived 19 residue peptide binds as a single amphipathic a-helix, into a hydrophobic surface channel on the C-terminal helical domain. The Axin-binding channel is formed by an α -helix (262-273), and an extended loop from 285-299 which was poorly ordered in the unphosphorylated GSK3β structure but is well defined in the Axin-peptide complex. On one wall of the channel, a hydrophobic helical ridge formed by Axin residues Phe388, Leu392, Leu396 and Val399 packs into a helical groove formed by Val263, Leu266, Val267 and Ile270 of GSK3β. On the opposite wall of the channel Pro385, Ala389, Ile393 and Leu396 of Axin pack against Tyr288, Phe291, Phe293, Pro294 and Ile296 from the extended GSK3β loop. The base of the channel at the N-terminal end of the Axin peptide is formed by the side chains of Ile228 and Phe229, which pack against the side chain of Phe388. In addition to the substantial hydrophobic interface, there are a few polar side chain interactions: Arg395 and Gln400 of the Axin peptide makes hydrogen bond with Asp264, and Gln295 of GSK3β[18]. Although the Axin-GSK3β structure has been nearly clarified, the possibility of molecular target for Axin-GSK3β binding sites had not been verified yet.

The purpose of the present study was to examine the interaction of Wnt co-receptor LRP6 with Axin2-GSK3β complex during Wnt signaling has been activated and clarify the specific binding site at the Axin2-GSK3β interaction through the mutations of GSK3β-binding residues derived from Axin2 and Axin2-binding residues derived from GSK3β. This study, therefore, is aimed to verify the possibility of molecular target for Axin-GSK3β binding sites as anti-EMT drug candidates.

Ⅱ. MATERIALS AND METHODS

1. Cell culture

The A549, HCT116, SW480, 293 and MCF7-Axin2 inducible cell lines were cultured in Dulbecco's modified Eagle's medium(DMEM) supplemented with 10% fetal bovine serum(FBS), 100 units/ml penicillin, and 100 g/ml streptomycin at 37℃ with 5% CO_2.

2. Plasmid DNA

shRNA duplex for human Axin2 and LRP6 knockdown and scrambled negative control duplex were purchased from OligoEngine. HA-tagged GSK3β was a gift from J.R.Woodgett(Ontario Cancer Institute, Ontario, Canada). Amino-terminal-deleted GSK3β were generated by polymerase chain reaction(PCR) using a HA-tagged GSK3β cDNA as a template, followed by subcloning into the pCDNA3 expression vector (Invitrogen) with *Eco*RI and *Xba*I sites. Expression vectors for amino-terminal-deleted GSK3β mutants : GSK3β-Δ9, GSK3β-K85M, GSK3β-K85R, GSK3β-L128A, GSK3β-Y216F, GSK3β-VE267/268GR, and GSK3β-F291L. His-tagged human Axin2 was subcloned into pCI vector with with *Eco*RI and *Xba*I sites. Amino-terminal-mutanted Axin2 were generated by polymerase chain reaction(PCR) using a His-tagged human Axin2 cDNA as a template, followed by subcloning into the pCI expression vector with *Eco*RI and *Xba*I sites. Expression vectors for amino-terminal-deleted Axin2 mutants : Axin2-L374E,

Axin2-L374G, Axin2-R377E, Axin2-378E, Axin2-378G. His tagged Axin2 sequence from pCI-Axin2-His vector was subcloned into episomal doxycycline-inducible expression vector.

3. Transfection

For LRP6 or Axin2 knockdown, cells were transfected with siRNA duplex(10pmol). 293 cells were cotransfected with 1.2ug GSK3β mutants and 0.3ug Axin2 expression vector. A549 cells were transfected with 1.5ug Axin2 mutants. MCF-7 cells were transfected with episomal Axin2 expression vector and selected with 200ug/ml of hygromycin for stable transfectant. The day before transfection plate cells into 12 mm dish so that they are 80~90% confluent the day of transfection. One tube was diluted with 3 μg DNAs into OptiMEM and mixed gently. The other tube was diluted with 4 μl Lipofectamine 2000 (Invitrogen) into 100 μl OptiMEM and incubated within 5 min at room temperature. Diluted DNAs and Lipofectamine reagent was combined and mixed gently and incubated for 30 min at room temperature to allow DNA-loposome complexes to form. While complex were forming, the medium on the cells was replaced with 1 ml DMEM without penicillin. For each transfection, cells were added with 600 μl medium contains complexes dropwise onto the cells and incubated for 4~6 h at 37°C in a CO2 incubator. Following incubation, cells were added with 2 ml DMEM contains 10% FBS into plate without removing the transfection mixture. The medium was replaced with fresh, complete medium at 18~24 h following the start of transfection, and the cells were harvested.

4. Immunoblot analysis

Whole cell or nuclear extracts prepared using a nondenaturing buffer (10 mM HEPES at pH 7.5, 150 mM NaC1, 2 mM EDTA, 2mM EGTA, 1 % NP-40, 4 mM PMSF). These were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes and incubated with anti-Snail polyclonal antibody, anti-HA (Roche, Indianapolis, IN), anti-GSK3β (BD Bioscience), anti-E-cadherin (Zymed), anti-tubulin (Ab Frontier) or anti-HDAC1 (Sigma) as indicated in the text. Bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies and the ECL system(Intron, Korea).

5. RT-PCR

mRNA was purified from the indicated cell lines using the RNEasy kit (QIAGEN) according to the manufacturer' recommended protocol. 2g RNA was added to RT-PCR reactions containing the indicated primers at a concentration of 0.6 M. After a 55 cycles, 30 min reverse transcription step, Axin2 mRNA was PCR amplified in 32 cycles for 1 min at each of the following temperatures: 94, 62, and 72C. PCR products were analyzed on 1% agarose gels (Table I).

Table I. The primers used in the study.

Type	Base Sequence of Primer	condition
Axin2	5‘-CTCTTCCCAAAGCCAGAGTG-3' 5'-CAGCGTCAACACCATCATTC-3'	60℃, 30cycles
GAPDH	5'-TGAAGGTCGGAGTCAACGGATTT-3' 5'-CATGTGGGCCATGAGGTCCACCAC-3'	58℃, 28cycles

6. Immunoprecipitation

Whole cell Triton X-100 lysates were incubated with Ni-NTA agarose (Qiagen) at 4°C for 2 hours and washed with lysis buffer. MCF7 Axin2 inducible cell lysates were incubated with 10uM chemical into Ni-NTA agarose at 4°C for 4 hours. The proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose, and bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies and the ECL system (Intron, Korea). For detecting endogenous GSK3β, and E-cadherin, nuclear and cytoplasmic fractions (prepared according to the manufacturer’ protocol; NucBuster Protein Extraction Kit, Novagen) were prepared from cells. Following SDS-PAGE, proteins were detected with either an anti-HA (Roche, Indianapolis, IN), anti-Conductin (Santacruz), anti-FLAG (Sigma), anti-Tubulin (Lab Frontier), anti-GSKβ (BD Bioscience), anti-Ecadherin (Zymed Laboratories Inc.), or anti-HDAC (Sigma) antiserum.

7. Chemical screening

Structure-based virtual screening through conformational and docking analyses was performed by Bioinformatics & Molecular Design Research Center. The distinctive binding model for active molecules was proposed based on docking studies with the binding site of Axin2-GSK3β complex. The new ligands candidates were selected which are surrogates of Axin peptide for blocking Axin2-GSK3β interactions.

8. Transwell migration assay

HCT116 cells were cultured and treated with #27 chemical candidate for 24 h. Then the cells were trypsinized and seeded at 1 x 10^5 cells per well into trans-well inserts (3.0μm pore, BD bio sciences) for migration assays. The wells were washed with PBS and fixed with 70% ethanol after 18h for migration assays. The cells on the apical side of each insert were then scraped off with cotton. The number of cells that had migrated to the basal side of the membrane and bottom of culture plates were visualized by 0.5% crystal violet stain. Pictures of 5 random fields from three replicate wells were obtained and the number of cells that had quantified nuclei of the cells migrated.

9. Reporter gene assay

HCT116 and SW480 cells were cotransfected with 1.0 g of reporter gene

Ecad(108)-Luc or Ecad(108)/EboxA.MUT/EboxB.MUT/EboxC.MUT-Luc and SV40-*Renilla* using Lipofectamine 2000 (Invitrogen). After 24hr, cells were incubated with 10uM of compound. Cell were lysised at 48h after chemical treatment and measued with the dual-luciferase assay system (Promega). T도 entire reporter assay was normalized by SV40-*Renilla*.

III. RESULTS

1. Alignment of Axin1 and Axin2.

Recent studies have demonstrated that, Axin1 has the GSK3β binding domain of nucleo-cytoplasmic shuttling. A sequence alignment was performed on the amino acid sequences of Axin1 and Axin2 from human gene. Residues with red boxes indicate those residues that overlap the position of GSK3β binding site between Axin1 and Axin2. Axin2 contains GSK3β nucleo-cytoplamic shuttling domain similar to that described for Axin1.

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346 YRI--RKQHRREMQESVQVNGRVPLPHIPRTVRVPKEV-RVEPQKFAEEL| 26946_sp1
325 YRVGSKKQLQREMHRSVKANGQVSLPHFPRTHRLPKEMTPVEPATFAAEL| 26946_sp2
    YR@  +KQ jREMjjSVj NGjV.LPHoPRT R@PKE@  VEP  FA EL

393 IHRLEAVQRTREAEEKLEERLKRVRMEEEGEDGDPSSGPPGPCHKLPPAP| 26946_sp1
375 ISRLEKLKLELESRHSLEERLQQIREDEEREGSELTLNSREG------AP| 26946_sp2
    I RLE @j   E.jj LEERLjj@R _EE E ._ .  .  .     AP

443 AWHHFPPRCVDMGCAGLRDAHEENPESILDEHVQRVLRTPGRQSPG----| 26946_sp1
419 TQHPLSLL--------PSGSVEEDPQTILDDHLSRVLKTPGCQSPGVGRV| 26946_sp2
    . H o.             . EE_P_.ILD_H@ RVL+TPG QSPG

489 -PGHRSPDSGHVAKM---PVALGGAASGHGKHVPKSGAKLDAAGL---HH| 26946_sp1
461 SPRSRSPDHHHHHHSQVHSLLPPGGKLPPAAASPGACPLLGGKGFVTKQT| 26946_sp2
     P  RSPD  H  +    .@  .G.  . .   P . . L . Go   j

532 HRHVHHHV--HHSTARPKEQVEAEATRRAQSSFAWGLEPHSHGARSRGYS| 26946_sp1
511 TKHVHHHVIHHHAVPKTKEEIEAEATQRVHCFCPGGSEYVCY-SKCKSHS| 26946_sp2
     +HVHHHo  HH. .+.KE_@EAEATjR j  o. G E     .+ +. S
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Table II. Sequence alignment of Axin1 and Axin2.

2. Regulation of Axin-GSK3β complex by LRP6

To verify Axin2-regulated GSK3β nucleocytoplasmic localization, HCT116 cells were transfected with Axin2-specific siRNA or non-silencing siRNA control. In Axin2 repressed conditions, the Axin2-mediated nuclear export function of GSK3β was blocked, and then GSK3β was accumulated in the nucleus. Consequently, nuclear Snail was phosphorylated by GSK3β and E-cadherin was increased inversely (A). To evaluate the regulatory role of LRP6 in Axin2-GSK3β complex, the protein levels of GSK3β and Snail were detected in nuclear fractions of A549 cells with LRP6-specific siRNA or non-silencing siRNA control. In the presence of an LRP6 siRNA, GSK3β was not exported from the nuclear compartment in a coincident fashion with Axin2 siRNA, and Snail protein level was decreased relative to control (B).

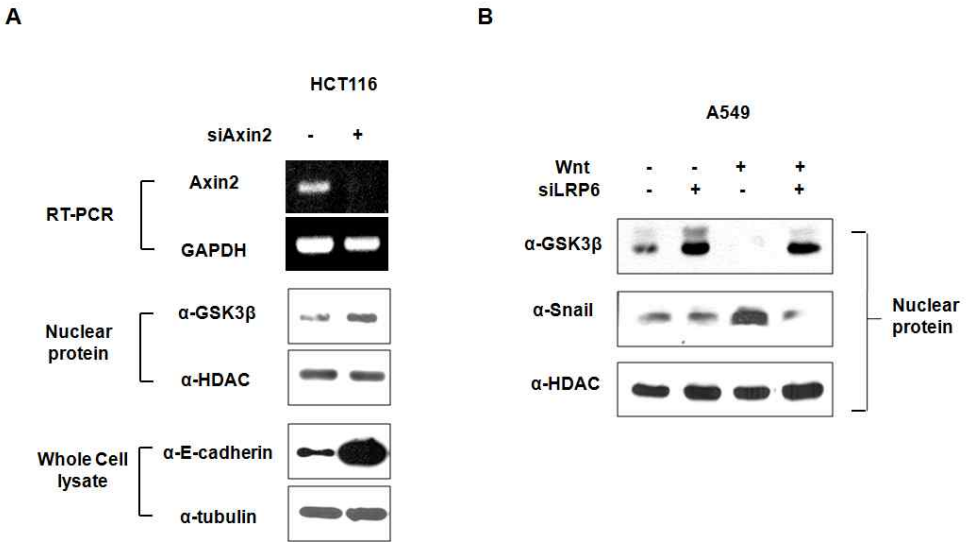


Fig. 2. Regulation of GSK3β nuclear compartmentalization by LRP6 and Axin2. (A) HCT116 cells were transfected with Axin2-specific or control siRNA expression vectors. Axin2 levels determined by RT-PCR. GSK3β levels in nuclear extracts were determined by immunoblot analysis. E-cadherin levels were determined in whole cell lysates. (B) Endogenous GSK3β and snail protein levels were detected by immunoblot in nuclear fractions of A549 with LRP6-specific or control siRNA expression vectors.

3. The binding sites in GSK3β on Axin2-GSK3β interaction

Since the previous studies suggested that GSK3β forms a low affinity homodimer that is disrupted by it's binding to Axin, the Axin2-GSK3β interaction was investigated with GSK3β mutants. The GSK3β with point mutations were initially used for analyzing Axin2-GSK3β interaction in a precipitation assay. Cells were cotransfected with HA-tagged human GSK3β (wild-type GSK3β, GSK3βΔ9, GSK3βK85M, GSK3βK85R, GSK3βL128A, GSK3βY216F, GSK3βVE267/268GR, GSK3βF291L) and Flag-tagged human Axin2 expression vectors. Co-immunoprecipitation of Axin2 expression vector and GSK3β mutants was performed to delineate the GSK3β sequences involved in binding to Axin2. GSK3βΔ9, GSK3βK85R, GSK3βL128A and GSK3βF291L mutants showed similar binding affinity with wild type GSK3β. While the K85M mutant showed negligible binding affinity to Axin2. The binding affinity of K85R mutant of GSK3β and Axin2 was similar to wild type. Mutants of V267E and G268R failed to bind with Axin2. Both protein expression levels GSK3β and Axin2 were determined in whole cell lysates.

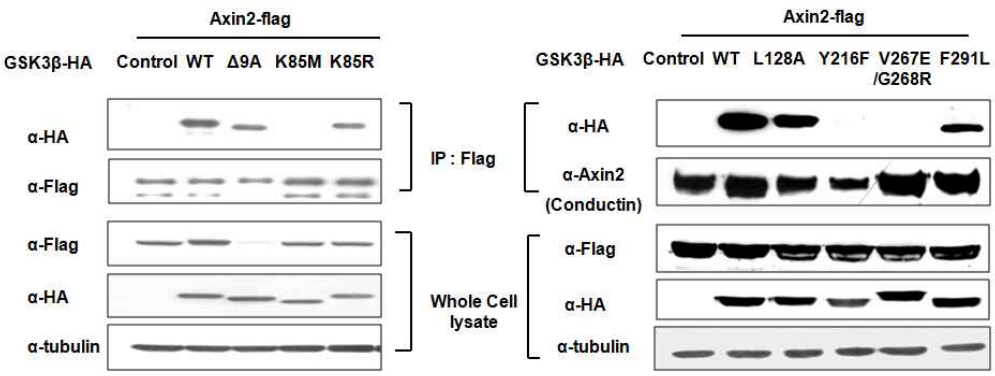
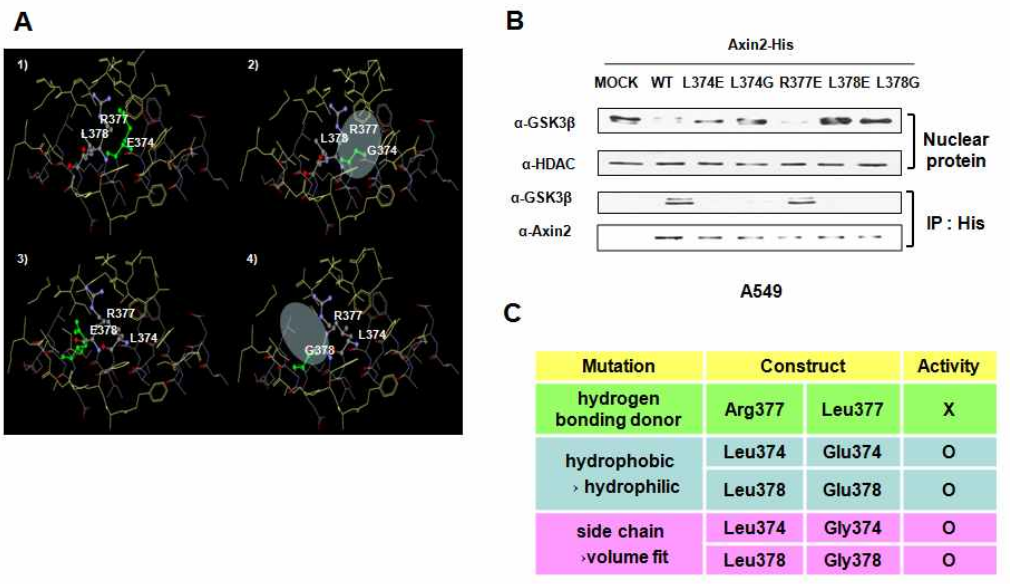


Fig. 3. GSK3β mutants on Axin2 binding. 293 cells were cotransfected with wild-type GSK3β or GSK3β mutants and Axin2 expression vectors. Axin2 and GSK3β expression levels determined in whole cell lysates. Protein complexes were immunoprecipitated with Flag-bead and detected by immunoblot analysis using anti-HA and anti-Flag antibodies.

4. The binding sites in Axin2 on Axin2-GSK3β interaction

To identify binding sites on Axin2 that mediated the interactions with GSK3, the several point mutations were generated predominantly at the Axin2 molecule. The structural specificity of Axin2 binding sites and the key amino acid residues of Axin2 interacting with GSK3 have been predicted by the analysis of X-ray crystal structure of GSK3-Axin2 complex.

Through surface structural assay and immunoblot assay, Axin2 mutants in Leu374 and Leu378 residues, which formed hydrophobic bond with GSK3β, showed altered binding affinity with GSK3β and its localization. Both the hydrophilic change only (Leu to Glu) or volumetric change (Leu to Gly) of Leu374 or Leu378 in Axin2 residues were effective. By contrast, the R377E mutant, which lost the function of hydrogen bond donor, showed no change of Axin2-GSK3β interactions.



Mutation	Construct		Activity
hydrogen bonding donor	Arg377	Leu377	X
hydrophobic › hydrophilic	Leu374	Glu374	O
	Leu378	Glu378	O
side chain ›volume fit	Leu374	Gly374	O
	Leu378	Gly378	O

Fig. 4. Contribution of Axin2 mutants to binding with GSK3β and its localization. (A) Axin2 mutants on the three-dimensional surface of GSK3β. Three-dimensional structure of GSK3β with the bound Axin2 mutant peptide. 1) Axin2-L374E, 2) Axin2-L374G, 3) Axin2-378E, 4) Axin2-378G. (B) A549 cells were transfected with wild-type Axin2 or Axin2 mutants expression vectors. Axin2 dependent GSK3β localization was determined in nuclear protein. Binding affinity to GSK3β in Axin2 mutants were determined by immunoprecipitation and immunoblot analysis. (C) Summary of activity of Axin2 mutants.

5. Effects of Axin2 inhibitors on Axin2-GSK3β interaction

To develop a molecular target that could inhibit Axin2-mediated nuclear GSK3β export function by inhibition of Axin2-GSK3β interaction, The structure-based virtual screening were performed for developing of the inhibitors of Axin2-GSK3β interaction, and 85 candidates were selected. in vitro inhibition potencies of candidates toward Axin2-GSK3β interaction was examined by immunoprecipitation and immunoblot analysis using lysates of His-tagged Axin2 expressing cell line. Lysates of the purified His-tagged Axin2 were incubated with 10uM and precipitated by the addition of His affinity resin. Among them, 18 compounds showed the inhibitory effect of Axin2-GSK3β interaction (A). To determine the endogeneous binding inhibitory effect of 18 compounds, the compounds were treated in cancer cell lines. It was observed that the endogenous nuclear GSK3β levels were increased in A549 cancer cells.

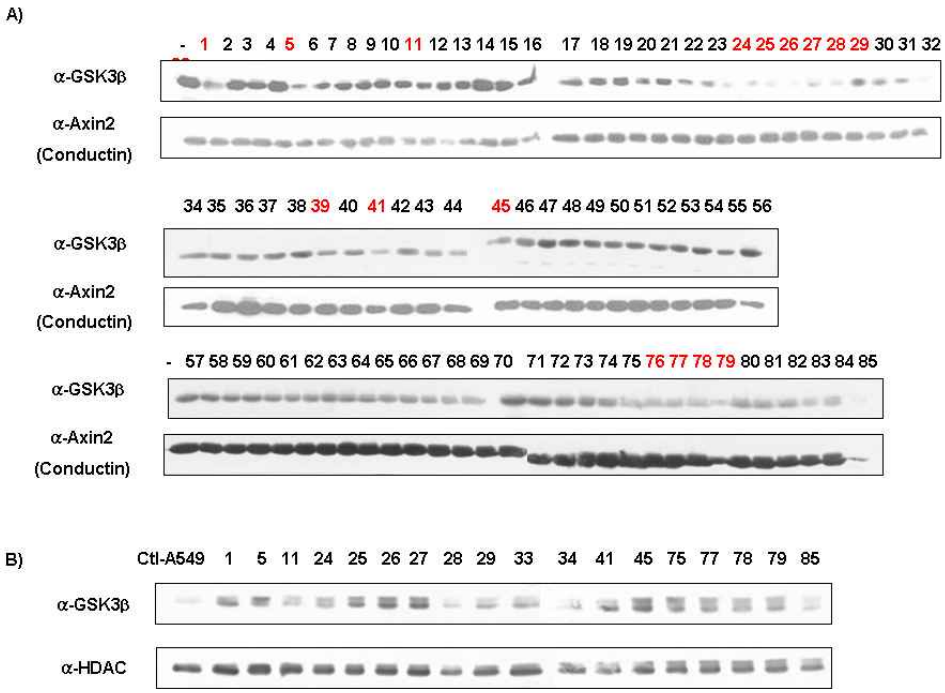


Fig. 5. Screening of chemical candidates blocking Axin2-GSK3β interaction. Binding affinity to GSK3β against compounds were determined by immunoprecipitation and immunoblot analysis. (A) Lysates of the purified His-tagged Axin2 expressing cell lines were incubated with 10uM for 3hrs. The samples were precipitated by the addition of His affinity resin and detected by immunoblot using anti-GSK3β antibody. (B) A549 was incubated with 18 compounds at a final concentration of 10uM. GSK3β nuclear levels were determined by immunoblot using anti-GSK3β.

6. Effect of Axin2 inhibitor on Wnt signaling

A molecular target (#27) could superiorly inhibit Axin2-mediated nuclear GSK3β export function by inhibition of Axin2-GSK3β interaction. The target molecule of Axin-GSK3β interaction increased nuclear GSK3β expression relative to control. Snail and E-cadherin expression levels were detected in whole cell lysates by immunoblot analysis. Endogenous Snail was suppressed and consequently E-cadherin expression was increased. The effect of chemicals on E-cadherin promoter activity were assessed with the reporter construct, Ecad(-108)-wt and Ecad(-108)-Mut, which harbors mutations in all three E-boxes, and immunoblot analysis. The target chemical increased E-cadherin promoter activity. Ultimately the chemicals markedly inhibited cancer cell migration in vitro transwell migration assay. By contrast, treatment with control (DMSO) neither affected E-cadherin promoter activity or invasion.

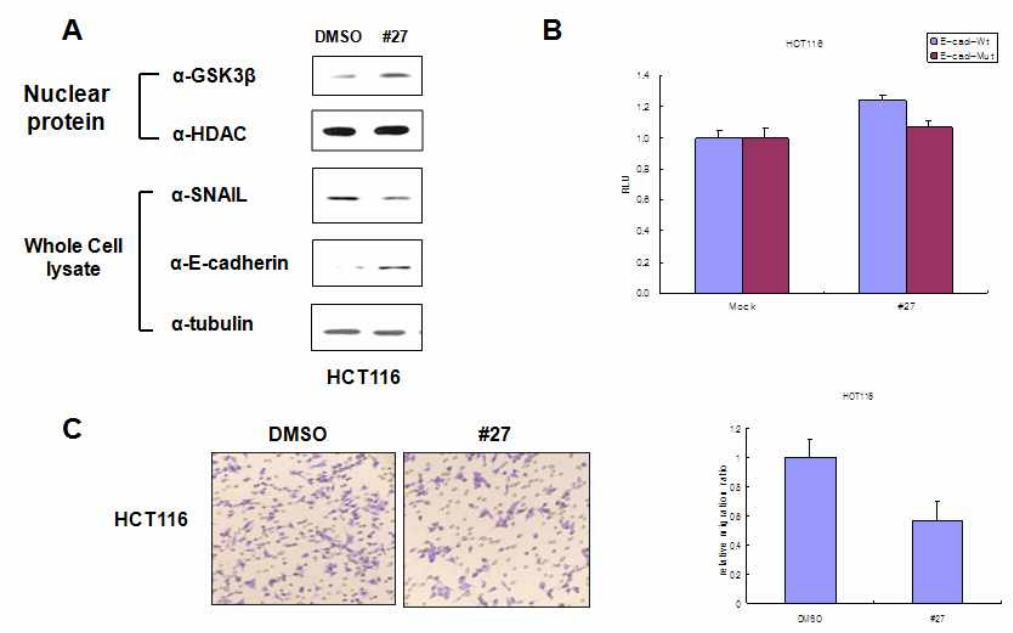


Fig. 6. Blocking of GSK3β nuclear export and cancer cell migration by Axin inhibitor. (A) HCT116 cells were incubated in the absence or chemical (10uM) for 16hr. Endogenous GSK3β, Snail, E-cadherin levels were detected by immunoblot analysis. (B) The chemical effect on E-cadherin promoter activity were assessed with the reporter construct, Ecad(-108)-wt and Ecad(-108)-Mut, which harbors mutations in all three E-boxes. (C) Representative images of transwell migration assay with the absent or chemical 10uM for 16hr. Cells were stained with 0.25% crystal violet. Data summary of migration assay.

IV. DISCUSSION

Recent studies indicate a reciprocal functional linkage between Wnt/β-catenin-mediated transcriptional regulation and Snail-mediated EMT. The accumulated nuclear β-catenin binds to TCF/LEF transcriptional factor and activates various target genes, including c-myc, Cyclin D1, matrilysin/MMP-7, PPARδ, and c-kit[10,14,15]. Axin2 is also one of the downstream targets regulated by β-catenin[19-21]. Among the various Wnt/β-catenin target genes, Axin2 is the only regulatory component of the Wnt signaling itself. The previous studies proposed that Axin2 may function as negative regulator of Wnt signaling, because Axin2 can act as a scaffolding protein that mediates the GSK3β-dependent phosphorylation of degradation of β-catenin[22]. It is not until Axin2 is recently demonstrated to serve a nuclear export function of GSK3β that Axin is regarded as oncogene, which can act as potent inhibitors of the phosphorylation of alternate GSK3β targets[23]. Regardless of the relative roles that Axin2 play in regulating Wnt responsiveness, Axin2 will only induce EMT under conditions where Snail1 transcription has been activated[14]. Given the ability of Snail1 to affect proliferation, apoptosis, migration and invasion in neoplastic cell populations[24], the canonical Wnt-dependent regulation of Axin2 may function as an important axis for regulating EMT programmes in cancerous states. The cytoplasmic tail portion of Wnt co-receptor, LRP5/6 recruits Axin2-GSK-3β complex to the membrane, which facilitates Axin2-regulated GSK3β nuclear export according to the present study. It is demonstrated that not only Wnt receptor Fz binds with Dvl, but Dvl and Axin2 also bind each other in cell membraneous portion[25,26]. Although the

activated Wnt signaling might enhance the binding affinity between these signal transductions, the regulatory mechanism of Fz-Dvl complex and their roles in nuclear GSK3β compartmentalization had not been clarified yet.

The binding and interaction of Axin-GSK3β is a crucial process in Wnt signaling pathway, which occurs independent of β-catenin. Based on the known crystal structure of Axin1-GSK3β complex, the surface structure of Axin2-GSK3β complex was investigated and the critical binding sites were decided at the Axin2-GSK3β interaction. Interestingly, Axin1 and Axin2 share a number of structural and functional properties, including homologous GSK3β -binding domains[1,5,6]. Compared the sequence alignment between Axin1 and Axin2, Axin2 has the site in consistent with the known specific site of Axin1 that binds GSK3β. Based on both structural analysis and immunoblot with both Axin2 and GSK3β mutants, it was demonstrated that GSK3β residue Y216 and V267/268, and Hydrophobic amino acid residues of Axin2, L374 and L378 were crucial sites to form principal Axin2-GSK3β protein-protein interaction. Intriguingly, the removal of hydrogen bond donor through Arg to Leu mutation in Axin2 residue Arg377 had no effect on the Axin2-GSK3β interactions.

The well-characterized interaction between GSK3β and Axin provided an essential molecular target. It was expected that inhibitors of this interaction could prevent the GSK3β nuclear export function of Axin and the interaction between GSK3β and some of its substrates and priming kinases, and therefore reorientate the overall cellular GSK3β activity towards other substrates. New chemicals as potential antagonists of Axin2-GSK3β interactions, selected by virtual screening and in vitro binding assay, showed potent antagonism to the

action of Axin2. The candidate molecule targeting Axin-GSKβ interaction showed effective inhibition of nucleocytoplasmic GSK3β export and cancer cell migration.

GSK3β is regulated at multiple levels, and it would be helpful to understand the regulatory function of GSK3β because the previous various efforts to develop selective and potent inhibitors for GSK3 would give us an information about Axin-GSKβ target[27-30]. First, GSK3β is known to be regulated by post-translational phosphorylation of Ser9 (inhibitory) and Tyr216 (activating); Ser21and Tyr279 respectively in GSK-3α[31]. (Wang). Phosphorylated Ser9 in the N-terminal domain of GSK-3β acts as a pseudo-substrate that blocks the access of substrates to the catalytic site. Unphosphorylated Tyr216 in the T-loop domain of GSK-3β prevents access of substrates to the catalytic site, but it's phosphorylation enables the substrate to be phosphorylation by GSK-3β [32]. Second, GSK-3β is regulated by interactions with many other proteins. Axin and presenilin act as docking proteins that allow the substrates to make contact with the priming kinase and GSK-3β[33], and docking proteins might specify different GSK-3β functions in the cell. To develop the therapeutic drug targeting GSK-3β, thus an drug effect on both the priming phosphorylation site and Axin-binding site in GSK-3β should be inspected[34,35]. Third, GSK3β action requires the priming phosphorylation of its substrates by another kinase on a serine residue located four amino acids C-terminal to the GSK3β phosphorylation site[36,37]. Fourth, GSK3β is regulated through its intracellular distribution[38]. The binding between Axin and GSK3β, is necessarily required to phosphorylate Axin, APC and β-catenin and to activate their complex. Therefore, inhibition of Axin-GSK3β binding, or in fact any other interactions

within the complex, could be a far more specific way to regulate this pathway.

GSK3β is an essential protein kinase that regulates numerous functions within the cell. Therefore, it is necessary to develop chemical drugs which can inhibit the specific binding site of Axin-GSK3β for targeting Wnt pathway. These chemicals could effectively block cancer invasion and metastasis through the regulation of the Wnt pathway with the minimal influence over other signaling pathway. The novel drugs can be also applied for other various diseases associated with Wnt signaling pathway asa well as anti-EMT drug.

Ⅴ. CONCLUSION

1. LRP6 stabilizes Snail and sustains its nuclear accumulation by Ain2-dependent nuclear export of GSK3β.

2. Amino acid residues of Y216 and V267/268 in GSK3β is crucial binding sites to interact with Axin2.

3. Hydrophobic amino acid residues of Axin2, L374 and L378 form principal Axin2-GSK3β protein-protein interaction, but a single hydrogen bonding residue R377 is not critical for Axin2-GSK3β binding.

4. The target molecule of Axin-GSK interaction increases nuclear GSK3β expression and activity, consequently inhibits Snail expression. The target also represses E-cadherin expression, and inhibits cancer cell migration.

The results of the study clarified the functional regulation of GSK3β nucleocytoplasmic compartmentalization mediated by LRP5/6 and Axin2, and verified the possibility of molecular target for Axin-GSK3β binding sites as anti-EMT target.

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ABSTRACT(IN KOREAN)

Axin2-GSK3β 복합체 결합부위의 분자 특성화

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임성미

암환자의 90% 이상이 상피 기원의 암종이며, 상피간엽이행 현상은 암의 초기 단계에서 침습성 암종으로의 진행 과정에 연관되어 있다. Wnt 신호전달은 Snail의 인산화를 억제시키는데, 이러한 과정은 Axin2에 의한 GSK3β의 핵외 수송을 통해 일어나며 이로 인해 E-cadherin 억제인자인 Snail 발현이 증가되고 상피간엽이행이 유도된다. 이 과정에서 LRP5/6를 통한 Axin의 활성화가 Wnt 신호 전달에 필수적인 것으로 알려져 있으나, LRP5/6를 통한 nuclear GSK3 comparmentalization에 대해서는 거의 알려져 있지 않다. 또한 최근에 알려진 Axin과 GSK3β의 결합 구조에 따르면, Axin과 GSK3β 간에는 다수의 소수성 결합과 하나의 수소 결합이 있으며, Axin에서 나선형의 'ridge'를 이루는 소수성 아미노산 잔기 부위가 GSK3β의 나선형 구조와 연결된 루프 구조 사이에 형성된 소수성의 'groove' 부위로 접혀 들어가는 형태로 결합이 일어난다. 그러므로 LRP5/6를 경유하는 Wnt 신호 전달 및 EMT 유도과정에서 Axin과 GSK3의 결합 부위는 Snail 발현을 조절 할 수 있는 분자 표적으로서 가능성이 있으나 이에 대한 연구는 거의 없다. 따라서 본 연구는 LRP5/6에 의한 GSK3 nuclear compartmentalization 조절과 그 과정에서 Axin의 역할을 규명하고, 또한 Axin2-GSK3β 단백질 간 결합에 관여하는 주요 분자 결합부위를 파악하고 이를 타겟하는 화합물을 이용하여 Axin2-GSK3β complex의 치료 타겟으로서의 가능성을 알아보고자 하였다. 결과는 다음과 같다.

1. LRP6 보조 수용체는 Snail을 안정화시키고 핵 내 농도를 증가시키는데, 이는 Axin2에 의한 GSK3β의 nuclear export function에 의존한다.

2. GSK3β mutants를 이용한 in vitro binding assay 결과, GSK3β 의 Y216 and V267/268 부위가 Axin2와의 결합에 중요한 부위이다.

3. Axin2의 소수성 아미노산 잔기인, L374와 L378부위가 Axin2-GSK3β 단백간 상호작용에 중요한 부위이며, 수소결합 부위인R377부위는 Axin2-GSK3β 결합에 결정적이지 않았다.

4. Pharmacophore 모델을 통해 선택된 Axin2-GSK3β결합억제 선도물질은 핵 내 GSK3β 발현을 증가시키고, Snail은 억제시켰다. 또한 E-cahderin 발현을 증가시키고 암세포의 이동을 억제시켰다.

이상의 결과에서 GSK3β의 nucleocytoplasmic compartmentalization의 조절에 LRP5/6 보조수용체와 Axin2가 관여함을 알 수 있었고, Axin2-GSK3β 결합의 부위는 상피간엽이행을 억제하는 타겟으로 적절함을 알 수 있었다.

핵심어 : LRP5/6, Axin2, GSK3β, 핵외 수송, Wnt 신호전달, 상피간엽이행