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Silybin Synergizes with Wnt3a in Activation of the Wnt/β-catenin Signaling Pathway through Stabilization of Intracellular β-Catenin Protein

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The Wnt/β-catenin signaling pathway regulates diverse developmental processes and adult tissue homeostasis. Inappropriate regulation of this pathway has been associated with human diseases, such as cancers, osteoporosis, and Alzheimer's disease. Using a cell-based chemical screening with natural compounds, we discovered silybin, a plant flavonoid isolated from the *Silybum marianum*, which activated the Wnt/β-catenin signaling pathway in a synergy with Wnt3a-conditioned medium (Wnt3a-CM). In the presence of Wnt3a-CM, silybin up-regulated β-catenin response transcription (CRT) in HEK293-FL reporter cells and 3T3-L1 preadipocytes through stabilization of intracellular β-catenin protein. Silybin and Wnt3a-CM synergistically reduced expression of important adipocyte marker genes including peroxisome-proliferator-activated receptorγ (PPARγ) and CAATT enhancer-binding protein α (C/EBPα) in 3T3-L1 preadipocytes, accompanied by the activation of Wnt/β-catenin signaling pathway. Taken together, our findings indicate that silybin is a small-molecule synergist of the Wnt/β-catenin signaling pathway and can be used as a controllable reagent for investigating biological processes that involve the Wnt/β-catenin signaling pathway.

Keywords: Wnt/β-catenin pathway, silybin, synergist

Introduction

The Wnt family consists of at least 19 cystein-reich secrected glycoproteins that control diverse developmental processes, such as cell proliferation, cell polarity, cell migration and cell differentiation [1, 21, 31]. Wnts bind to Frizzled (Fz) receptors and co-receptors to initiate distinct signaling pathways classified as either canonical or non-canonical pathways [8, 18, 30]. The canonical Wnt pathway, also known as the Wnt/β-catenin pathway, is controlled by cytoplasmic β-catenin levels. In the absence of a Wnt signal, cytoplamsic β-catenin forms a complex with adenomatous polyposis coli (APC) and Axin, which facilitate its phosphorylation by casein kinase 1 (CK1) and glycogen synthase kinase-3β (GSK-3β), resulting in the degradation of β-catenin via a ubiquitin-dependent mechanism [1, 12]. However, upon Wnt interaction with Frizzled (Fz) receptor and low-density lipoprotein receptor-related protein5/6 (LRP5/6) co-receptor, the recruitment of Dishevelled (Dvl) to Fz leads to LRP5/6 phosphorylation and then induces association of Axin complex with phosphorylated LRP5/6, thereby inhibiting Axin-mediated β-catenin phosphorylation and stabilizing cytobplamic β-catenin [31]. This is followed by translocation to the nucleus, where β-catenin forms a complex with members of the T cell factor/lymphocyte enhancer factor (TCF/LEF) transcription factor families, which activates the expression of Wnt/β-catenin responsive genes [13, 14, 28, 29].

Cellular progression from preadipocyte to adipocyte, or adipogenesis, is a complex process involving coordinated changes in morphology, hormone sensitivity, and gene expression, and is regulated by a balance of local and endocrine factors [20, 27]. The molecular mechanisms that govern adipogenesis have been well investigated using immortalized preadipocyte cell lines such as 3T3-L1 and 3T3-F442A [4, 23, 24]. Stimuli such as glucocorticoid agonist, high concentrations of insulin, cAMP, CAATT enhancer-binding protein β (C/EBPβ), and C/EBPδ are rapidly and transiently up-regulated during the early stages

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of adipogenesis, which subsequently promotes the expression of the key adipogenic transcription factors, C/EBPα and peroxisome-proliferator-activated receptor γ (PPARγ). These factors synergistically induce the expression of various genes that are required for the adipocyte phenotype.

Silybin is the major active constituent of silymarin, an extract from the seeds of the milk thistle (*Silybum marianum*) and is known to provide a wide range of hepatoprotective effects, especially against diseases like hepatitis, cirrhosis and jaundice [15]. In addition, its antioxidant, anti-inflammatory, anti-carcinogenesis, anti-cancer activities were demonstrated in various *in vitro* and *in vivo* models against oxidative stress, inflammatory responses, and chemical carcinogen-induced tumor promotion [22]. Protective effect of silybin on UV-radiation-induced oxidative damage and carcinogenesis was also observed in skin cells and animal model [10, 16]. In the present study, we used chemical biology approach to identify silybin as a small-molecule synergist of the Wnt/β-catenin signaling pathway. Silybin activates Wnt/β-catenin signaling pathway in a synergy with Wnt3a *via* the stabilization of cytoplasmic β-catenin protein.

Materials and Methods

Cell culture, plasmid transfection, and luciferase assay

L cells that secrete Wnt3a, HEK293 cells, and 3T3L1 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 120 μg penicillin/mL, and 200 μg streptomycin/mL. The HEK293-FL and HEK293-SEAP reporter and control cell line was established as previously described [10, 25]. Wnt3a conditioned medium (Wnt3a CM) was prepared by culturing Wnt3a-secreting L cells in DMEM with 10% FBS for 4 days. The medium was harvested and sterilized using a 0.22-μm filter. Fresh medium was added, and the cells were cultured for 3 additional days. The medium was then collected and combined with the previous medium. The pTOPFlash and plasmids were obtained from Upstate Biotechnology (Lake Placid, NY, USA) and pCMV-RL were purchased from Promega (Madison, WI, USA. The transfections were carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Luciferase assays were performed using the Dual Luciferase Assay Kit (Promega).

Screening for natural compound synergister of Wnt/β-catenin signaling

The HEK293-FL reporter cells were inoculated into 96-well plates at 15,000 cells per well in duplicate and grown for 24 h. Next, Wnt3a-CM was added, and then the natural compounds were added to the wells. After 15 hours, the plates were assayed for firefly luciferase activity and cell viability.

Western blotting

The cytosolic fraction was prepared as previously described [7]. Proteins were separated by 4-12% gradient SDS-PAGE (Invitrogen) and transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked with 5% nonfat milk and probed with anti-β-catenin (BD Transduction Laboratories, Lexington, KY, USA), anti-C/EBPα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PPARγ (Santa Cruz Biotechnology), and anti-actin antibodies (Cell Signaling Technology, Beverly, MA, USA). After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology), and the bands were visualized using the ECL system (Santa Cruz Biotechnology).

Adipocyte differentiation

3T3-L1 preadipocytes were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 10% CO_2 and 37°C. After 2 days of confluence (day 0), cells were incubated for 2 days in DMEM supplemented with 10% FBS, 0.5 mM of 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 10 μg/mL insulin. Thereafter, medium was replaced every other day with DMEM containing 10% FBS and μg/mL insulin. BIM were treated into cells and supplemented at 2-day intervals when the culture medium was changed.

RNA extraction and semiquantitative RT-PCR

Total RNA was isolated with TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions, cDNA synthesis, reverse transcription, and PCR were performed as described previously [10]. The amplified DNA was separated on 1.5% agarose gels and stained with ethidium bromide.

Results

Identification of silybin as a synergist of Wnt/β-catenin signaling pathway

To identify cell permeable natural compounds that modulate the Wnt/β-catenin signaling pathway, we used HEK293-FL reporter cells that are stably harbored TOPFlash, a synthetic β-catenin/Tcf-dependent luciferase reporter, and the human Frizzled-1 (hFz-1) expression plasmids [10]. After the addition Wnt3a-conditioned medium (Wnt3a-CM) and each compound to HEK293-FL reporter cells, we detected firefly luciferase activity using a microplate reader (Fig. 1A). From the natural compounds screened, silybin was found to be the most potent Wnt3a synergist (Fig. 1B). Silybin activated β-catenin response transcription (CRT) in the presence of Wnt3a-CM in a dose-dependent manner, whereas it did not affect activity of FOPflash, a negative control reporter with mutated β-catenin/Tcf binding elements, in HEK293 control cells (Fig. 2A). Interestingly, silybin did not increased TOPFlash activity in the absence of Wnt3a-CM (Fig. 2B). To further confirm the synergistic effect of silybin on the Wnt/β-catenin signaling pathway,

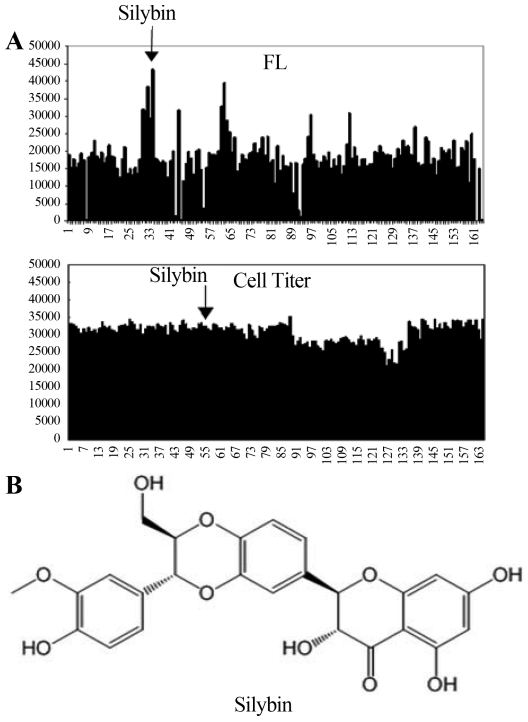

Fig. 1. Identification of silybin as a small-molecule synergist of Wnt/β-catenin signaling. (A) Screening of compounds that up-regulate Wnt/β-catenin signaling in the presence of Wnt3a-CM. (B) Chemical structure of silybin.

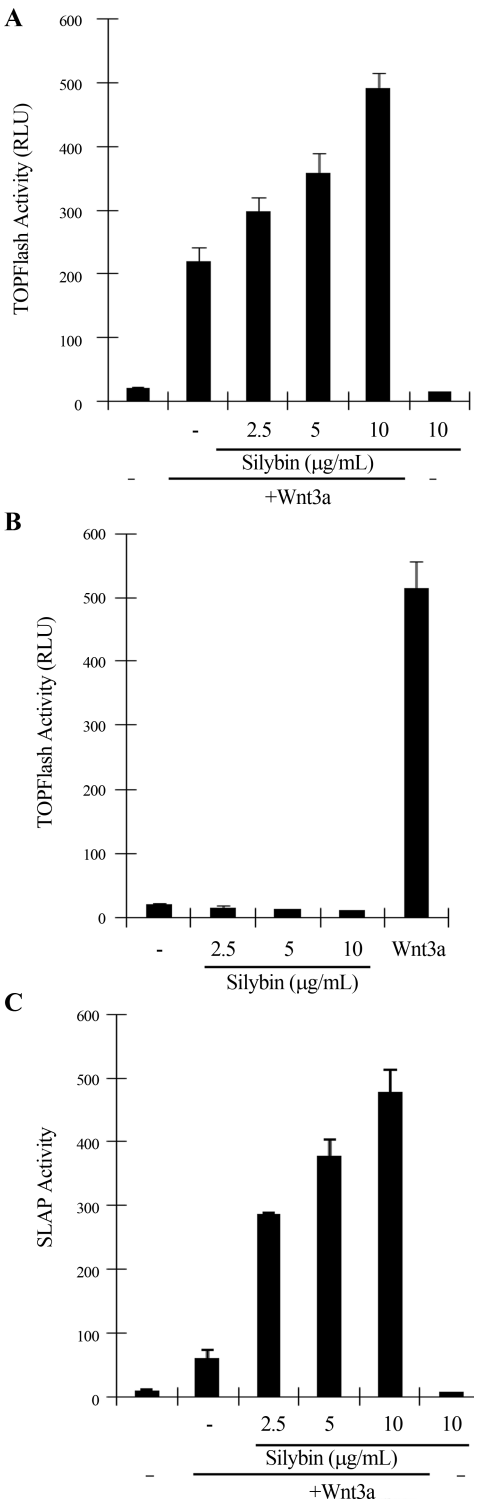

Fig. 2. Silybin activates Wnt/β-catenin signaling in the presence of Wnt3a-CM. (A, B) Compounds modulating TOPFlash reporter activity were screened using the HEK293 reporter cells. The controls were assayed in the presence or absence of Wnt3a CM. (C) SEAP activity of HEK293 reporter cells that incubated with indicated concentrations of silybin in the presence of Wnt3a-CM. TOPFlash activities were normalized with Celltiter-Glo activity.

we used other HEK293-SEAP reporter cells, which were stably transfected with a synthetic β-catenin/Tcf-dependent secreted alkaline phosphatase (SEAP) reporter and hFz-1 expression plasmids [25]. Consistent with results from the luciferase reporter assay, silybin synergized with Wnt3a-CM to induce SEAP activity in a dose-dependent manner (Fig. 2C). Taken together, these results suggest that silybin specifically activates the Wnt/β-catenin signaling pathway in a synergy with Wnt3a.

Silybin stabilizes cytoplasmic β-catenin protein in a synergy with Wnt3a

Within the Wnt/β-catenin signaling pathway, β-catenin response transcription is largely dependent on the level of cytoplasmic β-catenin, which is regulated by ubiquitin-dependent proteasome pathway [11]. To investigate whether silybin affects the intracellular β-catenin level, we used Western blot analysis with an anti-β-catenin antibody to determine the amount of cytosolic β-catenin in silybin-treated HEK293-FL reporter cells. As shown in Fig. 3A, in the presence of Wnt3a-CM, silybin increased β-catenin levels in cytoplasm, whereas silybin did not affect cytoplasmic β-catenin level without Wnt3a-CM, which is consistent with its effect on CRT. Interestingly, the β-catenin mRNA level did not change in response to different concentrations of silybin in the presence or absence of Wnt3a-CM in HEK293-FL reporter cells (Fig. 3B). These results suggest that silybin in a synergy with Wnt3a activates the Wnt/β-catenin signaling pathway via the up-regulation of β-catenin protein stability.

Silybin down-regulates the expression of C/EBPα and PPARγ in preadipocyte

Activation of the Wnt/β-catenin signaling pathway represses the expression of such master adipogenic transcription factors as peroxisome-proliferator-activated receptorγ (PPARγ) and CAATT enhancer-binding protein α (C/EBPα) [2, 5]. Given that silybin induces the accumulation of cytoplasmic β-catenin in HEK293-FL reporter cells in the presence of Wnt3a-CM, we hypothesized that sylibin and Wnt3a-CM synergistically inhibits the expression of C/EBPα and PPARγ. To test this hypothesis, we first examined whether 3T3-L1 preadipocytes are capable of transducing Wnt/β-catenin signaling when treated with silybin. We found that treatment of 3T3-L1 preadipocytes with silybin showed a consistent and robust, concentration-dependent increase in TOPFlash reporter activity with Wnt3a-CM (Fig. 4). In addition, the intracellular β-catenin level, an indicator of the activation status of Wnt/β-catenin signaling pathway, was also synergistically increased by incubation

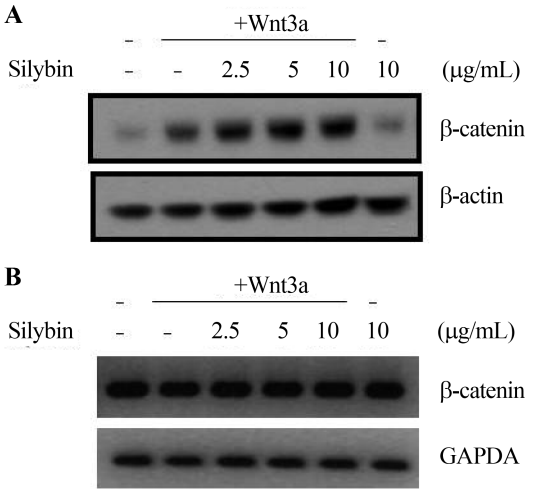

Fig. 3. Silybin stabilizes intracellular β-catenin protein in a synergy with Wnt3a-CM. (A) Cytosolic proteins were prepared from HEK293 cells treated with either vehicle (DMSO) or silybin (2.5, 5, 10 μg/mL) for 15 h and then subjected to western blotting with β-catenin antibody. The blots were re-probed with anti-β-actin antibody as a loading control. (B) Semiquantitative RT-PCR for β-catenin and GAPDH was performed with total RNA prepared from HEK293 reporter cells treated with the vehicle (DMSO) or silybin (2.5, 5, 10 μg/mL) for 15 h.

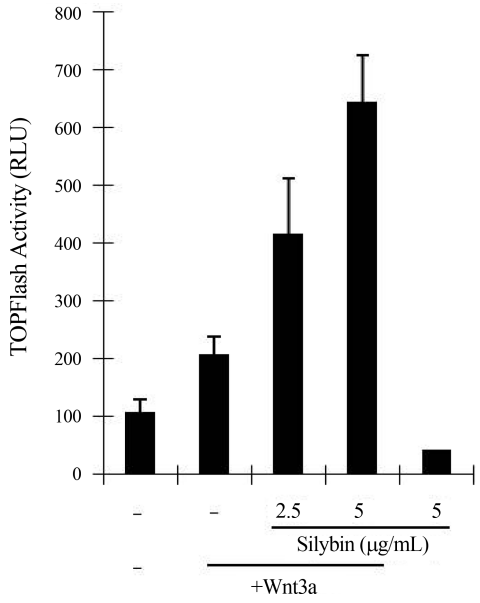

Fig. 4. Silybin activates Wnt/β-catenin signaling in the presence of Wnt3a-CM in 3T3L1 cells. 3T3L1 cells were co-transfected with TOPFlash at 24 h after transfection, increasing amounts of silybin (2.5, 5 μg/mL) were added to the transfected cells for 24 h, and the luciferase activity was measured.

with sylibin and Wnt3a-CM in 3T3-L1 preadipocytes (Fig. 5A). Finally, the β-catenin mRNA level consistently unchanged in response to different concentrations of silybin and Wnt3a-CM in 3T3-L1 preadipocytes (Fig. 5B). These results suggest that the Wnt/β-catenin signaling pathway can be activated in response to sylibin in 3T3-L1 preadipocytes.

To examine whether activation of the Wnt/β-catenin signaling pathway by silybin inhibits the expression of C/EBPα and PPARγ, 3T3-L1 preadipocytes were incubated with increasing amounts of silybin and Wnt3a-CM beginning at day 0 of the standard differentiation protocol. As shown in Fig. 6, silybin in a synergy with Wnt3a-CM significantly decreased the level of C/EBPα and PPARγ that was up-regulated by incubation with MDI. These results suggest that sylinbin synergizes with Wnt3a to inhibits adipogenesis by suppressing the expression of C/EBPα and PPARγ.

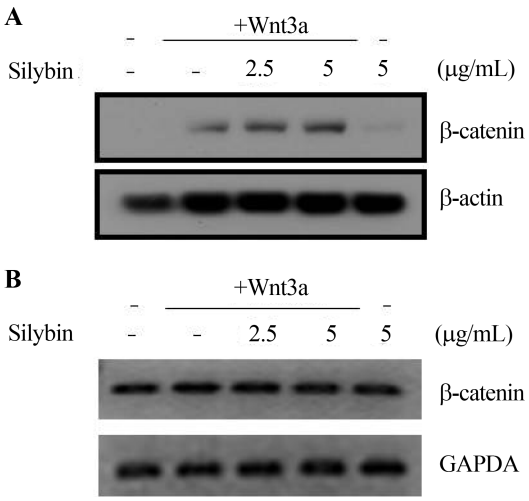

Fig. 5. Silybin stabilizes intracellular β-catenin protein in a synergy with Wnt3a-CM in 3T3L1 cells. (A) Cytosolic proteins were prepared from 3T3L1cells treated with vehicle (DMSO) or silybin (2.5, 5, 10 μg/mL) for western blotting with anti-β-catenin antibody. The blots were reprobed with anti-β-actin antibody as a loading control. (B) Semiquantitative RT-PCR for β-catenin and GAPDH was performed with total RNA prepared from 3T3L1 cells treated with the vehicle (DMSO) or silybin (2.5, 5, 10 μg/mL) for 15 h. The results are shown as the average of three experiments, the bars indicate standard deviations.

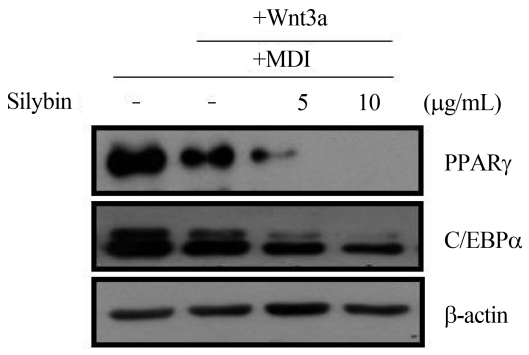

Fig. 6. Silybin decreases adipogenic gene expression in a synergy with Wnt3a. Total proteins were prepared from 3T3-L1 preadipocytes and then subjected to western blotting with anti-PPARγ and anti-C/EBPα antibodies. The blots were reprobed with anti-β-actin antibody as a loading control.

Discussion

The Wnt/β-catenin pathway is involved in the development and maintenance of many organs and tissues. The function of β-catenin, a key effector of the Wnt/β-catenin pathway, is determined by its N-terminal phosphorylation (Ser33/37), which is a prerequisite for its subsequent ubiquitination and degradation. In this study, a screen of natural compounds produced, which synergistically activated β-catenin response transcription (CRT) in a dose-dependent manner in the presence of Wnt3a. A number of small molecules have been identified that activate the Wnt/β-catenin signaling pathway by inhibiting GSK-3β activity, including 6-bromoindirubin-3'-oxime (BIO), CHIR99021, and LiCl [3, 17, 26]. Since GSK-3β is involved in multiple signaling pathways other than Wnt/β-catenin signaling, GSK-3 β inhibitors could have unexpected effects in the cell and organism models. Pyrimidine analogs, whose molecular targets and mechanisms of action are still unknown, have been identified as agonists of the Wnt/β-catenin pathway [19]. In addition, QS11 modulates the Wnt/β-catenin signaling pathway through GTPase activating protein of ADP-ribosylation factor (ARFGAP) [32]. Since silybin activates the Wnt/β-catenin signaling pathway only in the presence of Wnt3a, it would function through mechanism that cross-talk with Wnt/β-catenin signaling pathway and thus provide new insights into this pathway.

Previous studies implicate the Wnt/β-catenin signaling pathway in the regulation of adipocyte differentiation [3]. Ectopic expression of Wnt1 or Wnt10b in 3T3 cells induces the accumulation of cytoplasmic β-catenin and suppresses adipocyte differentiation [3]. In addition, pharmacological treatments (*e.g.,* LiCl or CHIR99021) that mimic the activation of Wnt/β-catenin signaling pathway also repress adipogenesis [33]. Conversely, inhibiting the Wnt/β-catenin signaling pathway by over-expressing Axin or dominant-

negative Tcf-4 in 3T3-L1 leads to spontaneous adipogenesis. In this study, silybin synergizes with Wnt3a to activate the Wnt/β-catenin signaling pathway in preadipocytes, and to induce accumulation of cytoplasmic β-catenin while suppressing adipocyte differentiation by blocking the expression of C/EBPα and PPARγ.

In conclusion, we used the cell-based natural compounds screening to identify silybin, a synergist of the Wnt/β-catenin signaling pathway, which inhibits the expression of C/EBPα and PPARγ in preadipocyte. Mutations in the Wnt-10b gene have recently been implicated in human obesity [6], and LRP5 polymorphisms have been shown to be significantly associated with an obese phenotype [9]. Taken together, our findings may facilitate the development of new therapeutics or preventive agents for obesity and its associated disorders.

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국문초록

Silybin에 의한 Wnt/β-catenin 신호전달체계의 활성화

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Wnt/β-catenin 신호전달체계는 세포의 분화와 증식, 기관의 발생과 조절을 담당하는 중요한 세포내 신호전달체계이다. 발생과정에서 Wnt/β-catenin 신호전달체계의 작용이 지방세포로의 분화를 억제하고 조골세포와 신경세포로의 분화는 촉진한다는 많은 연구들이 보고되어 있으며, 현재 Wnt/β-catenin 신호전달체계의 조절을 통한 여러 질병의 치료와 예방에 대한 관심이 대두되고 있다. 본 연구에서는 세포를 기반으로 한 초고속 저분자 스크리닝 시스템을 이용하여 Wnt의 상승제인 silybin을 발굴하였다. silybin은 Wnt가 존재 않을 경우에는 β-catenin 단백질의 수준에 영향을 미치지 않지만 Wnt가 존재할 경우, mRNA 발현양의 변화 없이 세포질내의 β-catenin 단백질의 수준을 증가시킨다. 또한 silybin에 의해 증가된 β-catenin으로 인해 지방세포분화에 중요한 전사인자라고 알려진 PPAR-γ와 C/EBP-α의 발현을 억제한다. 따라서 이 연구에서는 silybin이 세포질내 β-catenin 단백질의 수준을 증가시킴으로써 Wnt/β-catenin 신호전달체계를 활성한다는 사실을 제시하였다.