

Sox4 stimulates β -catenin activity through induction of CK2

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Abstract. β -catenin is a key component of the Wnt signaling pathway and the abnormal accumulation of β -catenin is characteristic of various types of cancer. Here we demonstrate that overexpression of Sox4 enhances β -catenin/TCF activity by increasing the stability of β -catenin. Sox4 increased the protein level of β -catenin and its target gene cyclin D1 in a dose-dependent manner. An siRNA experiment for Sox4 also demonstrated that Sox4 increases the protein levels of β -catenin and thus activates the Wnt signaling pathway. We found that induction of β -catenin/TCF activity by Sox4 is caused by stabilization of the β -catenin protein, but not by induction of β -catenin transcription. We further demonstrate that the increased level of β -catenin is caused by induction of CK2. In light of recent evidence that Sox4 expression is activated in the colon and in other tumors with β -catenin dysregulation, our findings suggest that Sox4 acts as an agonist of Wnt signaling in cancer cells.

Introduction

β -catenin plays a dual role in cells as a major structural component of cell-cell adherens junctions and as a pivotal signaling molecule in the Wnt signaling pathway. Wnt signaling plays a central role in regulation of cellular proliferation, differentiation, and morphogenesis, and is dependent on tightly regulated control of β -catenin stability (1-3). When active and present in a multi-protein complex containing adenomatous polyposis coli (APC) and Axin1 and/or Axin2 (also known as Axil or conductin), glycogen synthase 3 β (GSK3 β) can phosphorylate specific serine and/or threonine residues near the β -catenin N-terminus (3-5). The phosphorylated form of β -catenin is ubiquitinated and degraded by the proteasome (2,3,6). Upon Wnt signaling, β -catenin is stabilized and translocated to the nucleus, where it can bind

to members of the T cell factor (TCF)/lymphoid enhancer family (LEF) transcription factor family (7,8). In the nucleus, TCFs mediate sequence-specific DNA binding, and β -catenin, via its interaction with TCFs, activates transcription of target genes (1,9,10).

Defects that alter β -catenin regulation have been reported in various human cancers. Mutations in β -catenin gene (*CTNNB1*) sequences encoding crucial GSK3 β phosphorylation sites in the β -catenin N-terminal domain have been found in many different cancers (10). In upwards of ~80% of colorectal cancers, inactivation of the APC tumor suppressor gene is the predominant mechanism leading to β -catenin deregulation (1,11). In other cancers, mutations in the genes encoding one of the two Axin proteins have been reported (12-15). A prime consequence of mutational defects in β -catenin regulation is constitutive activation of downstream β -catenin-TCF-regulated target genes, including genes with major effects on cell growth regulation and tumorigenesis, such as *c-MYC* and *Cyclin D1* (16-18).

SRY-related HMG box (*Sox*) genes encode a family of transcription factors that are crucial for embryonic development. At least 30 members of the SOX family have been identified so far and are expressed in many different cell types and tissues at multiple stages during development (19-21). However, the precise function(s) of many SOX proteins is still unknown.

Sox4 was first identified as a transcription factor required for B- and T-lymphocyte differentiation (22). Sox4 appears to be critical for normal development and maturation of endocardial cushions and for normal B-cell maturation; however, its function(s) in these processes is not yet clear. Recent studies have demonstrated high expression of Sox4 in several tumors including breast cancer, colon cancer, salivary adenoid cystic carcinoma, and medulloblastomas, suggesting that Sox4 may play a role in tumorigenesis (23-29).

Of particular interest, several studies have suggested possible cross-talk between Sox and β -catenin by showing that several of the Sox proteins, such as Sox3, Sox7, Sox9, and Sox17 modulate Wnt signaling (30-34). Sinner *et al* recently showed that Sox17 represses Wnt signaling by promoting degradation of β -catenin and TCF via a GSK3 β -independent mechanism. They also reported that unlike Sox17, Sox4 enhances the Wnt signaling pathway (35). However, the precise action mechanism of Sox4 on β -catenin is unclear. In this study, we examined the effect of Sox4 on β -catenin and reported evidence showing that Sox4 stabilizes β -catenin by inducing the level of casein kinase 2 (CK2).

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Materials and methods

Cell culture and transfection. HEK 293 and SW480 cells were maintained at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal bovine serum. HEK 293 cells were transiently transfected with various expression vectors using FuGENE[®] HD (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. An electroporator (Neon[™] Transfection System, Invitrogen, Carlsbad, CA) was used for transfection of SW480 cells.

Plasmids. The complete open reading frame of Sox4 (1425 nucleotides) corresponding to GenBank accession number NM_003107 was amplified by PCR using human skeletal muscle cDNA as a template (Clontech, Mountain View, CA). Mammalian expression vectors for N-terminally FLAG-tagged Sox4 proteins were created by insertion of cDNA fragments into the 5' *Hind*III and 3' *Eco*RI sites of pCDNA3.1-FLAG. All constructs were confirmed by DNA sequencing. The reporter constructs pTOPFLASH, which contains three copies of an optimal TCF binding motif (CCTTTGATC), and pFOPFLASH, which contains three copies of a mutant motif (CCTTTGGCC), have been described (36). Plasmid pCH110 (GE Healthcare, Piscataway, NJ) contains a functional *LacZ* gene cloned downstream of a cytomegalovirus early-region promoter-enhancer element.

Immunoprecipitation and immunoblotting. For immunoprecipitation, SW480 cells were transiently transfected using an electroporator (Neon Transfection System, Invitrogen), according to the manufacturer's instructions. After 48 h, cells were washed twice with PBS and lysed in RIPA buffer (PBS supplemented with 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM PMSF, 1 μ g/ml aprotinin, and 1 mM sodium orthovanadate). Cell lysates were harvested and incubated at 4°C for 30 min and cleared by centrifugation at 10,000 \times g for 10 min. Supernatant was incubated with agarose-conjugated anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO) for 3 h. Immunoprecipitates were washed 4 times with RIPA buffer containing 0.05% SDS and boiled in SDS-PAGE sample buffer. For immunoblotting, proteins were resolved by SDS-PAGE and immunoblotted using antibodies against FLAG, actin (Sigma-Aldrich), β -catenin (Millipore, Bedford, MA), GSK3 β (BD Transduction Laboratories, Rockville, MD), phospho-GSK3 β (Cell Signaling Technology, Danvers, MA), CK2, cyclin D1, cyclin D3, and c-myc (Santa Cruz Biotechnology, Santa Cruz, CA).

Luciferase reporter gene assay. To assess the effects of Sox4 on endogenous β -catenin-induced TCF activity, SW480 cells (2 \times 10⁷ cells) were co-transfected with pTOPFLASH or pFOPFLASH, pCDNA3.1-FLAG-Sox4, and pCH110 using the Neon Transfection System (Invitrogen) and seeded on 12-well plate. The total mass of transfected DNA in each well was kept constant by addition of empty vector DNA, when necessary. After 48 h, cells were washed twice with PBS and lysed with Reporter Lysis Buffer (Promega, Madison, WI). Luciferase activity was measured using the Luciferase

Activity Assay kit, as described by the manufacturer (Promega). All experiments were performed in triplicate, and the mean \pm SD values were determined. β -galactosidase activities were determined in order to normalize the luciferase activities.

Semi-quantitative RT-PCR. Total RNA was isolated with TRIzol[®] reagent (Invitrogen). RT-PCR was conducted using the ONE-STEP RT-PCR PreMix kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. Primers used in the RT-PCR reaction are: β -catenin forward primer (5'-CACAGCTCCTCTGACAGAGTTACTTCACTC-3') and reverse primer (5'-CTCAGCTTGGTTAGTGTGTCAGGCA-3'); cyclin D1 forward primer (5'-CGTCTCGGGA GAGGATTAGGTTCC-3') and reverse primer (5'-CCA AGTAGCTGTGGGTTGAACCTG-3'); GAPDH forward primer (5'-CCAAGGTCATCCATGACAACTTTG-3'), and reverse primer (5'-GTCATACCAGGAAATGAGCTTG ACA-3'). RT-PCR was performed under the following cycle conditions: 1 cycle of 30 min at 45°C, 1 cycle of 5 min at 94°C, and 22 cycles of 30 sec at 94°C, 30 sec at 55°C (63°C for cyclin D1), and 50 sec at 72°C, with a final extension at 72°C for 5 min. PCR products were then electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining.

siRNA experiment. The Sox4 siRNA construct was obtained as Silencer[®] Pre-designed siRNA (Applied Biosystems, Foster City, CA). HEK 293 cells were transfected with 50 nM of siRNA using X-tremeGENE siRNA Transfection Reagent (Roche Molecular Biochemicals), according to the manufacturer's instructions. After 48 h, cells were harvested and cell lysates were separated by SDS-PAGE. The expression level of Sox4 was analyzed by immunoblotting, as described above.

Results

Sox4 increases the level of endogenous β -catenin. Among Sox family members, Sox4 is of great interest in respect to cancers because recent studies have provided evidence that Sox4 is involved in tumorigenesis of various human cancers (23-29). To examine the reciprocal relationship between Sox4 and β -catenin, HEK 293 cells were transfected with expression vectors for FLAG-tagged Sox4. Thirty hours after transfection, cells were lysed and Western blot analysis was performed using anti- β -catenin and anti-FLAG antibody. The result shown in Fig. 1A clearly demonstrate that the levels of endogenous β -catenin were increased with increasing concentrations of Sox4. In accordance with this result, when the cells were transfected with Sox4 siRNA, the level of β -catenin was decreased (Fig. 1B). Furthermore, Sox4 knockdown resulted in a significant reduction in the levels of c-myc and Cyclin D, typical targets of the Wnt signaling pathway, suggesting that Sox4 up-regulates the level of endogenous β -catenin.

Sox4 activates the Wnt signaling pathway by stabilizing the β -catenin protein. To further demonstrate whether Sox4 can affect the signaling activity of β -catenin, β -catenin/TCF transcriptional activity was measured using the pTOPFLASH

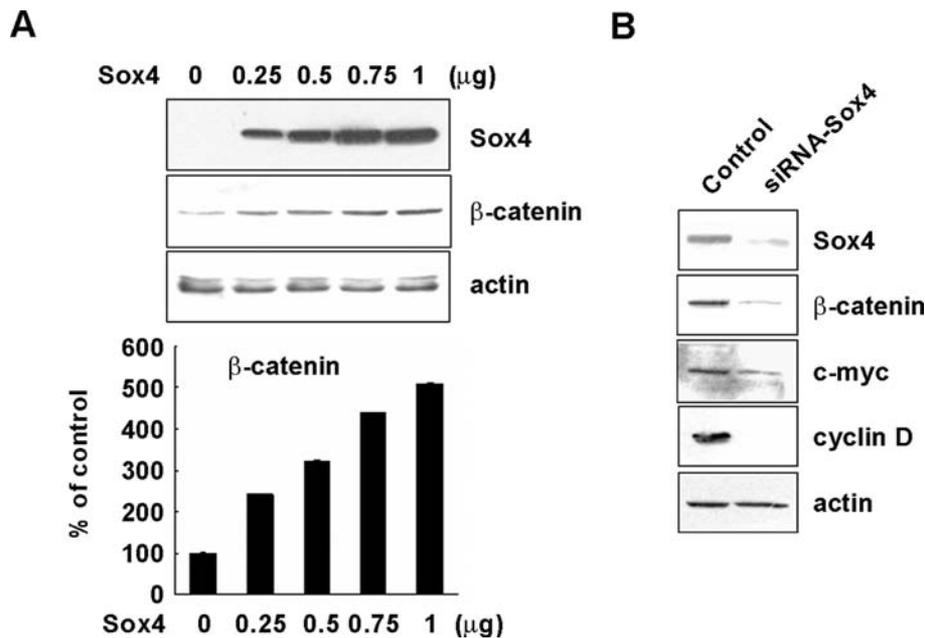


Figure 1. Sox4 up-regulates the levels of endogenous β -catenin. (A) HEK 293 cells were transfected with increasing amounts of FLAG-tagged Sox4 expression vectors. After 30 h, the levels of Sox4 and β -catenin were determined by Western blot analysis using anti-FLAG and anti- β -catenin antibodies. (B) HEK 293 cells were transfected with Sox4 siRNA constructs. After 48 h, the protein levels of Sox4, β -catenin, c-myc, Cyclin D, and actin were detected by Western blot analysis.

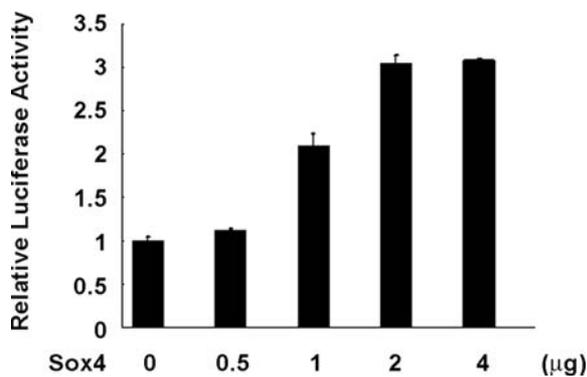


Figure 2. Sox4 induces β -catenin/TCF stimulated transcription. SW480 cells were co-transfected with the pTOPFLASH or pFOPFLASH reporter vector and expression vectors encoding Sox4, as indicated. pTOPFLASH contains three TCF binding sites upstream of a luciferase reporter and the pFOPFLASH plasmid has mutated TCF binding motifs. After 48 h, cell extracts were subjected to the luciferase assay. Luciferase activities were normalized for transfection efficiency by co-transfection with a β -galactosidase-expressing vector, pCH110. Data represent mean values from triplicate experiments.

luciferase reporter vector. pTOPFLASH contains three TCF consensus binding sites and is strongly activated by positive components of the Wnt signaling pathway (36). SW480 human colon adenocarcinoma cells were used for the reporter gene assay. As shown in Fig. 2, transfection of SW480 cells with Sox4 mammalian expression vector increased transactivation of the pTOPFLASH vector in a dose-dependent manner, suggesting that Sox4 activates the Wnt signaling pathway.

Endogenous protein levels can be regulated by RNA level and/or protein level. To understand the regulatory mechanism

of β -catenin by Sox4, SW480 cells were transfected with the Sox4 expression vector for 48 h and the protein and RNA levels of β -catenin were examined. As expected, when cells were transfected with the Sox4 expression vector, protein levels of β -catenin were increased depending on the Sox4 concentration, suggesting that Sox4 increases the endogenous protein level of β -catenin (Fig. 3A). Next, in order to verify the effect of Sox4 on β -catenin gene expression, we examined the mRNA level of β -catenin by semi-quantitative RT-PCR. Unlike protein level, mRNA level of β -catenin was not affected by Sox4 expression, suggesting that Sox4 did not affect the transcription level of β -catenin (Fig. 3B). Both mRNA level and protein level of Cyclin D were increased depending on the Sox4 concentration, demonstrating that Wnt signaling is activated by Sox4. These results suggest that the increased level of β -catenin by Sox4 is caused by increased protein stability, but not by transcriptional activity.

Sox4 stabilizes β -catenin by inducing the level of CK2. Targeting of β -catenin to the proteasome is achieved through its phosphorylation by GSK3 β in a multiprotein complex. According to our data, Sox4 up-regulated the protein level of β -catenin, but did not affect the mRNA level, suggesting that Sox4 may affect phosphorylation of β -catenin by GSK3 β and subsequent ubiquitination by β -TrCP. To verify involvement of GSK3 β on Sox4-mediated induction of β -catenin, cells were treated with LiCl, an inhibitor of GSK3 β activity. As shown in Fig. 4A, LiCl accumulated the endogenous β -catenin. Similar to results shown by cells treated with LiCl alone, overexpression of Sox4 also increased the level of β -catenin (Fig. 4A). This result suggests the possibility that Sox4 may increase the level of β -catenin by inhibition of GSK3 β activity. Therefore, we next examined whether Sox4 affects GSK3 β

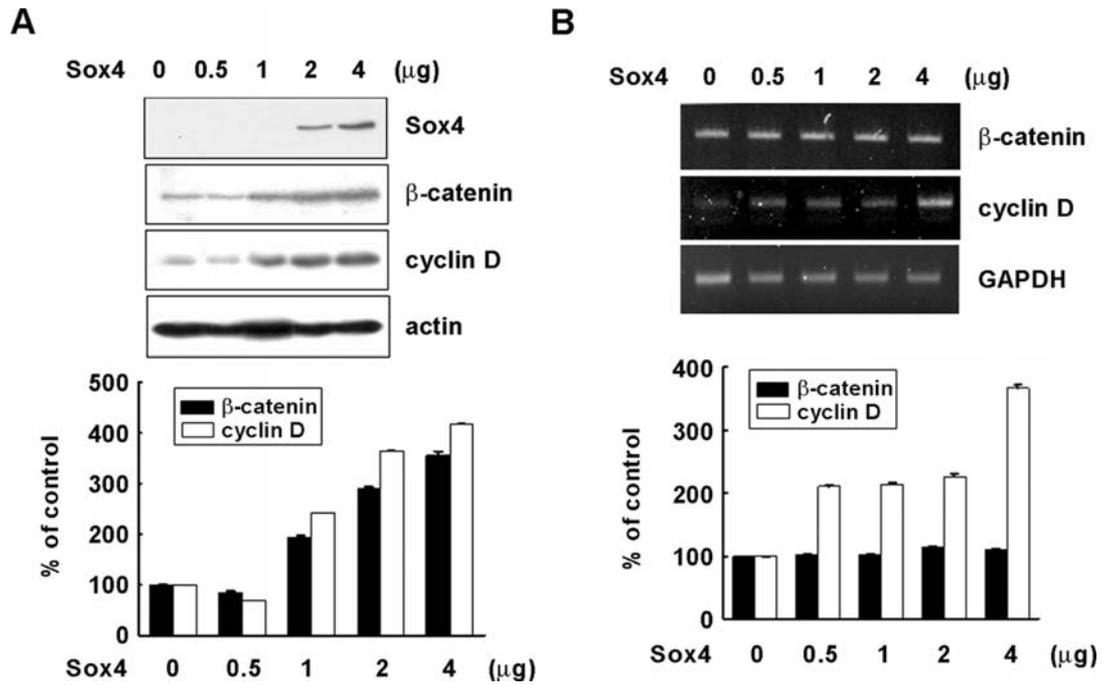


Figure 3. Sox4 up-regulates the protein level but not the mRNA level of β -catenin. SW480 cells were transfected with increasing amounts of FLAG-tagged Sox4 expression vectors. (A) After 48 h, the protein levels of Sox4, β -catenin, Cyclin D, and actin were detected by Western blot analysis. (B) After 48 h, total RAN was isolated and the expression levels of β -catenin, Cyclin D, and GAPDH were analyzed by semi-quantitative RT-PCR.

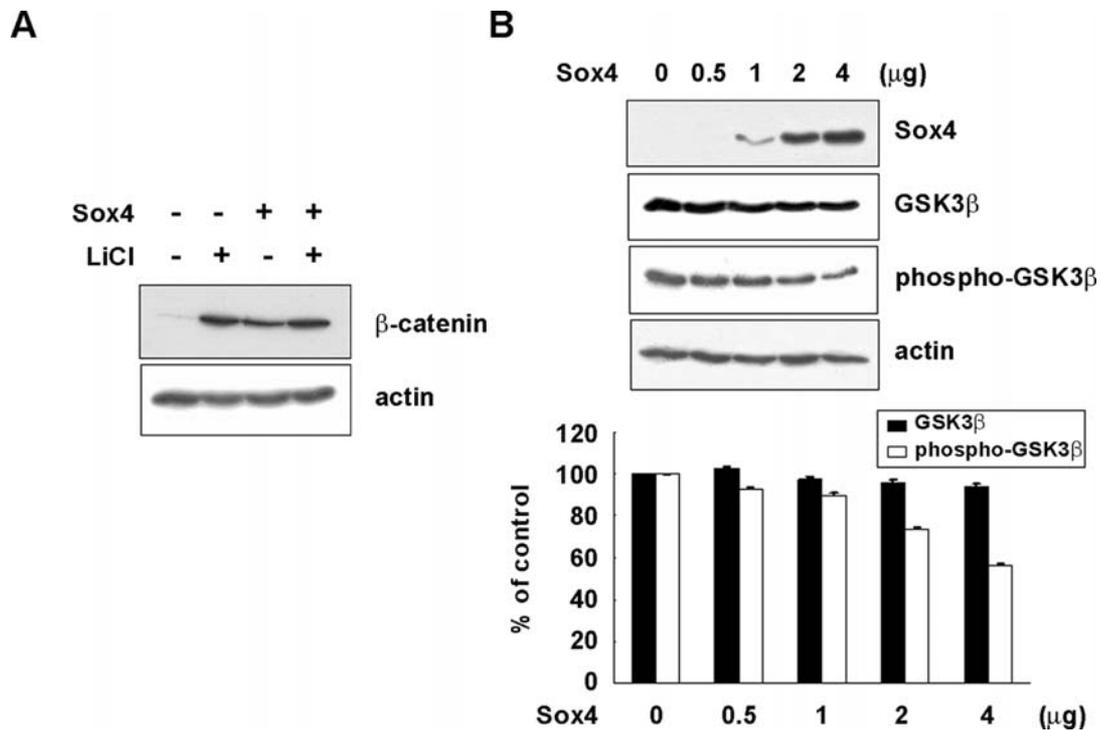


Figure 4. Sox4 slightly decreased the level of phosphorylated GSK3 β . (A) SW480 cells were transfected with the Sox4 expression vector. After 44 h, cells were treated with LiCl (30 mM) and incubated further 4 h. Cells were harvested and the level of β -catenin was determined by Western blot analysis. (B) SW480 cells were transfected with increasing amounts of Sox4 expression vectors. After 48 h, the levels of Sox4, total GSK3 β , phospho-GSK3 β , and actin were detected by Western blot analysis.

activity. SW480 cells were transfected with expression vectors for Sox4 and the levels of total and phosphorylated GSK3 β were examined. Unexpectedly, the level of total GSK3 β was not affected by Sox4. Moreover, phosphorylated

GSK3 β , an inactivated form of GSK3 β , was slightly decreased depending on the Sox4 concentration.

Since Sox4 did not inactivate GSK3 β , we next examined whether Sox4 can affect the activity of CK2, another major

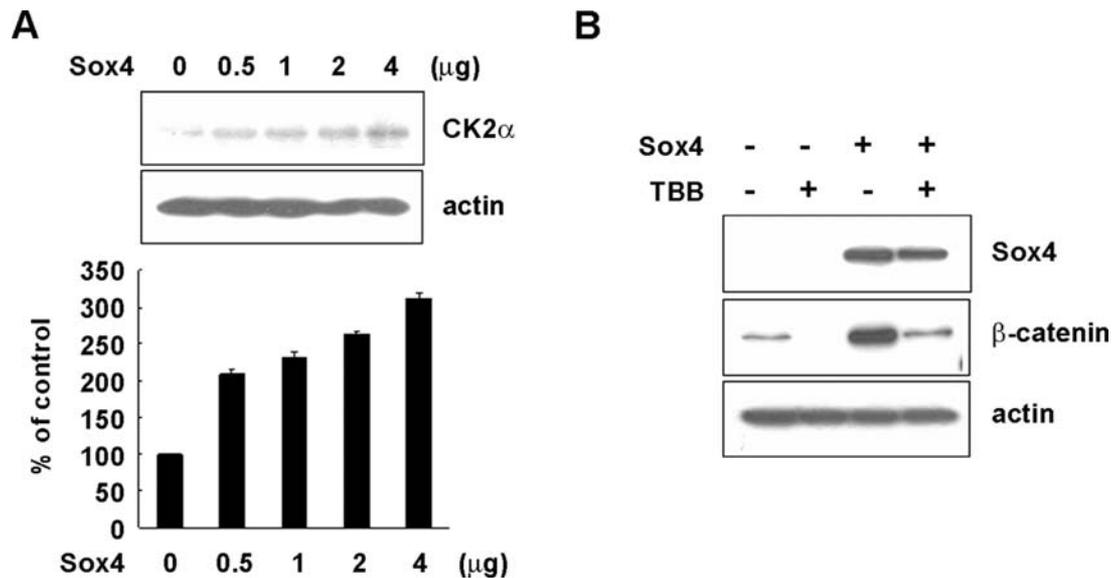


Figure 5. Sox4 increased the level of CK2. (A) SW480 cells were transfected with the Sox4 expression vector. After 48 h, cells were harvested and the level of CK2 α was determined by Western blot analysis. (B) SW480 cells were transfected with the Sox4 expression vector. After 24 h, cells were treated with TBB (100 μ M) and incubated for further 24 h. Cells were harvested and the level of Sox4 and β -catenin was detected by Western blot analysis.

kinase involved in regulation of β -catenin stability. Previous studies have reported that CK2 phosphorylates β -catenin and thereby prevents degradation mediated by the proteasome (37,38). Of particular interest, the level of endogenous CK2 was increased depending on the Sox4 concentration (Fig. 5A). When cells were treated with 4,5,6,7-tetrabromobenzotriazole (TBB), a specific inhibitor of CK2, the level of endogenous β -catenin was decreased (Fig. 5B). However, Sox4 compromise the effect of TBB on β -catenin, suggesting that Sox4 increased the level of endogenous β -catenin by increasing protein stability in a CK2-dependent manner.

Discussion

Increasing evidence has shown that Sox4 is up-regulated in several tumors, such as colon cancer, breast cancer, and salivary gland cancer (23-29). Although the precise molecular mechanism of Sox4 involvement in tumorigenesis is unclear, several members of the Sox family, including Sox3, Sox7, Sox9, and Sox17 have been shown to inhibit β -catenin activity (30-34).

In this study, we demonstrated that Sox4 up-regulates the endogenous level of β -catenin in both HEK293 and SW480 cells. RNAi experiments have also shown that depletion of Sox4 induced a decreased level of endogenous β -catenin and subsequent decrease of Wnt signaling target proteins, such as c-myc and Cyclin D. Endogenous protein levels can be regulated by RNA level and/or protein level. By showing that Sox4 did not affect the transcription level of β -catenin, we first demonstrated that Sox4 up-regulates endogenous β -catenin by increasing protein stability.

In the absence of the Wnt signal, β -catenin is phosphorylated by GSK3 β in its N-terminal domain, resulting in ubiquitination of β -catenin and its degradation by proteasome. Binding of Wnt to the receptor leads to inhibition of GSK3 β activity through mechanisms that have not been fully clarified. In this study, we demonstrated that Sox4 up-regulates the

amount of endogenous β -catenin by increasing protein stability. These results suggest the possibility that Sox4 may up-regulate β -catenin by regulation of GSK3 β activity. However, our results showed that the level of phosphorylated GSK3 β was slightly decreased while total GSK3 β level was not affected upon Sox4 overexpression. GSK3 β is a serine-threonine kinase and its active form is non-phosphorylated. Therefore, we ruled out GSK3 β as a factor that controls β -catenin stability by Sox4.

CK2 and GSK3 β are two important kinases which act oppositely on β -catenin stability. CK2 is a constitutively active serine-threonine kinase and is known to be regulated by the expression level. CK2 is up-regulated in most cancers and has been implicated in cellular transformation and development of tumorigenesis (38-40). Phosphorylation of the armadillo repeat region of β -catenin by CK2 has been reported to stabilize β -catenin and to protect it from proteasome degradation. Furthermore, overexpression of CK2 in transgenic mouse models has been reported to result in an increased β -catenin level, leading to development of mammary tumors (40). Here we first showed that CK2 is up-regulated depending on the Sox4 concentration. This finding confirms that Sox4 stimulates the Wnt signaling pathway by inhibiting degradation of the β -catenin protein via a CK2-dependent mechanism.

CK2 acts as a multisite regulator in the Wnt signaling pathway by phosphorylation and stabilization of both dishevelled and β -catenin. Dishevelled is a protein that acts directly downstream of the frizzled receptor. Phosphorylation of dishevelled by CK2 is believed to prevent GSK3 β from phosphorylation of β -catenin. In our results, Sox4 slightly activated GSK3 β , while the level of β -catenin was increased. This can be explained by the role of CK2, which phosphorylates the dishevelled.

In summary, we showed that Sox4 enhances β -catenin/TCF activity by increasing the stability of β -catenin. We first demonstrated that an induced level of endogenous β -catenin

by Sox4 is caused by increased protein stability, which is caused by induction of CK2. The exact molecular mechanism responsible for up-regulation of CK2 by Sox4 remains to be elucidated. However, our data provide a novel mechanism by which Sox4 acts as an oncogene by increasing endogenous β -catenin in a CK2-dependent manner. These findings represent a new molecular target for cancer therapy that targets the Wnt signaling pathway. Selective Sox4 inhibition or down-regulation in tumors may provide an opportunity for development of novel cancer drugs.

Acknowledgements

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