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KLF4 and SOX9 transcription factors antagonize β-catenin and inhibit TCF-activity in cancer cells

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ABSTRACT

The transcriptional activator β-catenin is a key mediator of the canonical Wnt signaling pathway. β-catenin itself does not bind DNA but functions *via* interaction with T-cell factor (TCF)/lymphoid-enhancing factor (LEF) transcription factors. Thus, in the case of active Wnt signaling, β-catenin, in cooperation with TCF/LEF proteins family, activates the expression of a wide variety of genes. To date, the list of established β-catenin interacting targets is far from complete. In this study, we aimed to establish the interaction between β-catenin and transcription factors that might affect TCF activity. We took advantage of EMSA, using TCF as a probe, to screen oligonucleotides known to bind specific transcription factors that might dislodge or antagonize β-catenin/TCF binding. We found that Sox9 and KLF4 antagonize β-catenin/TCF binding in HEK293, A549, SW480, and T47D cells. This inhibition of TCF binding was concentration-dependent and correlated to the *in vitro* TCF-luciferase functional assays. Overexpression of Sox9 and KLF4 transcription factors in cancer cells shows a concentration-dependent reduction of TCF-luciferase as well as the TCF-binding activities. In addition, we demonstrated that both Sox9 and KLF4 interact with β-catenin in an immunoprecipitation assay and reduce its binding to TCF4. Together, these results demonstrate that Sox9 and KLF4 transcription factors antagonize β-catenin/TCF in cancer cells.

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1. Introduction

Canonical Wnt/β-catenin signaling pathway is involved in controlling a wide range of developmental processes, including tissue patterning, cell fate, and cell proliferation [1,2]. Free β-catenin translocates to the nucleus, binds TCF proteins, and activates a multitude of genes [2,3]. The precise mechanism by which β-catenin/TCF activity is regulated is under extensive investigation but not yet solved. Although this mechanism has not been fully elucidated, there is evidence to support various possibilities such as protein–protein interaction, recruitment of co-activators or co-repressors by β-catenin and TCF [4], as well as β-catenin nuclear translocation [5].

The Krüppel-like factor (KLFs) family of gene regulatory proteins are transcription factors implicated in the regulation of a wide range of cellular processes, including proliferation, apoptosis, differentiation, inflammation, migration, and tumor formation [6–8]. Since cancers display uncontrolled cell growth, KLF4 is thought to play a key role in cancer progression and development [9,10]. KLF4 is proven to induce growth arrest [6,11] and to possess tumor suppressive activity [12,13]. This tumor suppressor activity of KLF4 is supported by *in vivo* evidence in which mice heterozygous for KLF4 manifest increased tumor burden when bred to the APC^{Min} mice that are genetically predisposed to intestinal adenoma formation [14]. KLF4 expression is shown to be downregulated in a number of cancers [13,15,16]. However, its role in cancer is not fully conclusive as it is also identified to act as an oncogene in some cancers and specifically breast cancer [17,18]. It is postulated that a cross talk between KLF4 and β-catenin regulates intestinal homeostasis [19]. Furthermore, it is deduced that KLF4 binds the transcriptional activation domain of β-catenin and inhibits its transcription [19]. A recent study showed that lower levels of KLF4 expression in the proliferative compartment of the intestinal epithelium are regulated by the transcription factors TCF4 and Sox9, an effector and a target, respectively, of Wnt/β-catenin [20,21]. This substantiates the finding that reduced levels of KLF4 tumor suppressor activity in colon tumors may be driven by elevated Wnt/β-catenin signaling. In addition, Yori et al. demonstrated that forced expression of KLF4 in the mice model inhibits primary tumor growth and metastasis of breast cancer cells [22]. These data support the role of KLF4 as a tumor and metastasis suppressor in the breast.

The SOX family of transcription factors has emerged as modulators of canonical Wnt/β-catenin signaling in diverse development and disease contexts [23] where they act as both agonists or antagonists of β-catenin/TCF activity [24]. Although Sox9 is required for differentiation of variety of tissues, its dysregulation results in intestine, lung, pancreatic, ovarian, and prostate cancer [25]. Sox9 is overexpressed in tumors from many origins [26], and from most data its anti-cancer effect was reported [27] in addition of decreasing Wnt signaling activity [28,29]. β-catenin has also been shown to interact with SOX-families

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and this interaction has been proposed as the mechanism by which Sox9 controls Wnt signaling in chondrocytes [30]. Conflicting results subsist regarding the mechanism by which Sox9 leads to β-catenin/TCF inactivation. While the association of Sox9 to the β-catenin destruction complex promotes β-catenin degradation in the nucleus [31], another study found that Sox9 inhibits β-catenin/TCF activity without affecting β-catenin level [19]. As Sox and TCF proteins bind similar DNA sequences [32,33], Sox proteins might also suppress Wnt-induced transcription by competing with TCF for the same promoter sites. However, *in vitro* DNA binding studies with optimized Sox and TCF DNA-binding sites argue against this model and suggest that Sox proteins bind an optimized TCF consensus sequence very poorly, if at all, and vice versa [23]. Furthermore, several members of the Sox family, including Sox17, Sox3, Sox7, and Sox9, have been implicated in repressing β-catenin activity by mechanisms that are not yet understood [34–37]. These studies along with others suggest that Sox9 and KLF4 proteins can cooperate with or antagonize β-catenin/TCF function depending on the context. In this study, we aimed to identify transcription factors that might interact with β-catenin to prevent its binding to TCF. This was achieved by visualizing protein-DNA binding using electrophoretic mobility shift assay and validating the obtained data with functional TCF-luciferase experiments. We found that among many transcription factors, Sox9 and KLF4 were the most potent in inhibiting β-catenin/TCF binding and activity.

2. Material and methods

2.1. Cell culture

Colon cancer cells (SW480, SW620, LoVo), breast cancer cells (T47D, MCF-9, Hs578T), and lung cancer cells (A549, H249, H460) were obtained from ATCC cell line collection (Manassas, VA). Cells were cultured in the same medium (DMEM) with 10% FBS to avoid any growth variability. HEK293 cells were purchased from ATCC and cultured in DMEM 10%. Cell passaging was performed when they reached 80% confluence and the medium is changed every three days.

2.2. Electrophoretic mobility shift assay (EMSA)

The oligonucleotides Sp1, AP1, NF-kB, CREB, TFIID, and OCT1 were purchased from Promega (Madison, WI) or synthesized from MWG Biotech (High Point, NC) (TCF, SRF, Sox9, KLF4, SREB, AP2, GATA, and NFAT). Nuclear extracts were prepared from cells using a nuclear and cytoplasmic extraction reagent (NE-PER, Thermo) following the protocol provided by the manufacturer. Oligonucleotides were dimerized and gel purified. Next, 10 μg of nuclear extract in 10 μl of binding buffer was mixed with 2 μg of poly[dI–dC] and 1 μg of BSA to a final volume of 19 μl. After 30 min incubation on ice, with or without excess of cold probes, 1 μl (50,000 cpm) of [γ-^{32}P]ATP (Perkin Elmer, Norwalk, CT) end-labeled double-stranded consensus oligonucleotides (either TCF, Sox9, or KLF4) was added to each reaction and incubated on ice for an additional 30 min. The reaction products were separated on a 6% native polyacrylamide 0.5% Tris–borate–EDTA gel. Gels were then dried and exposed to autoradiographic film. The sequences [5′→3′] of the oligonucleotides used in the EMSA are: TCF [GGTAAGATCAAAGGGG]; Sox9 [GGGAGAGAACAATGGGTGCC CTAC]; KLF4 [ATGCAGGAGAAAGAAGGGCGTAGTATCTACTAG]; GA TA [CACTTGATAACAGAAAGTGATAACTCT]; NF-κB [AGTTGAGGGGA CTTTCCCAGGC]; AP1 [CGCTTGATGAGTCAGCCGGAA]; CREB [AGA GATTGCCTGACGTCAGAGAGCTAG]; NFAT [CGCCCAAAGAGGAAAAT TTGTTTCATA]; Sp1 [ATTCGATCGGGGCGGGGCGAG]; AP2 [GATCGA ACTGACCGCCCGCGGCCCGT]; OCT-1 [TGTCGA ATGCAA ATCACTAG A A]; TFIID [GCAGAGCATATA AGGTGAGGTAGGA]; SRF [GGATGTC CATATTAGGACATCT]; and SREB [GCGTAGCTCTTTCTCCCCCCACCCA CCAACCT].

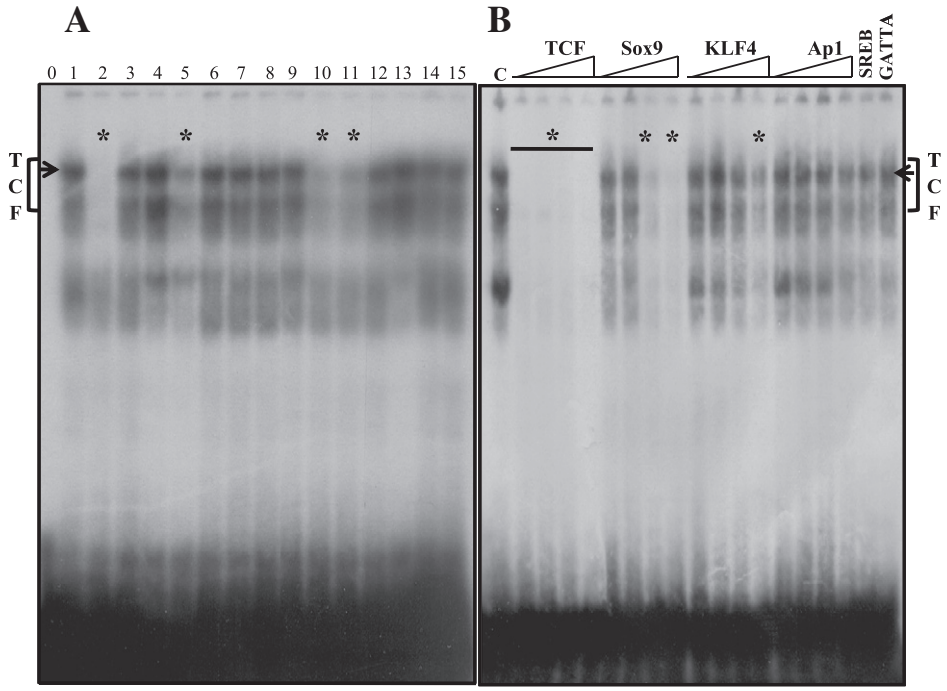


Fig. 1. Panel A. Competition of TCF-binding with different consensus oligonucleotide sequences. Nuclear extract proteins were prepared from SW480 colon cancer cells and EMSA were performed as described in the Material and methods. Excess of unlabeled oligonucleotides was preincubated with nuclear protein extracts (10 μg) 30 min before adding TCF-radiolabeled probe. After 30 min incubation in the presence of TCF-radiolabeled probe, proteins were resolved on native polyacrylamide gel. 0: free probe, 1: control, 2: TCF, 3: CREB, 4: Sp1, 5: Sox9, 6: NF-κB, 7: NFAT, 8: TFIID, 9: AP1, 10: KLF4, 11: SRF, 12: OCT1, 13: AP2, 14: SREB; 15, GATA. Panel B. The reduction of TCF binding by Sox9 and KLF4 oligonucleotides is concentration dependent. Nuclear extracts prepared from SW480 colon cancer cells were incubated with increasing amounts of TCF, Sox9, and KLF4 cold oligonucleotides for 30 min. TCF probe was added and incubation was carried for another 30 min before resolving proteins on native polyacrylamide gel. C: control, 1: 10-, 2: 25-, 3: 50-, 4: 100-fold excess in cold probes. Figures are representative of three independent experiments.

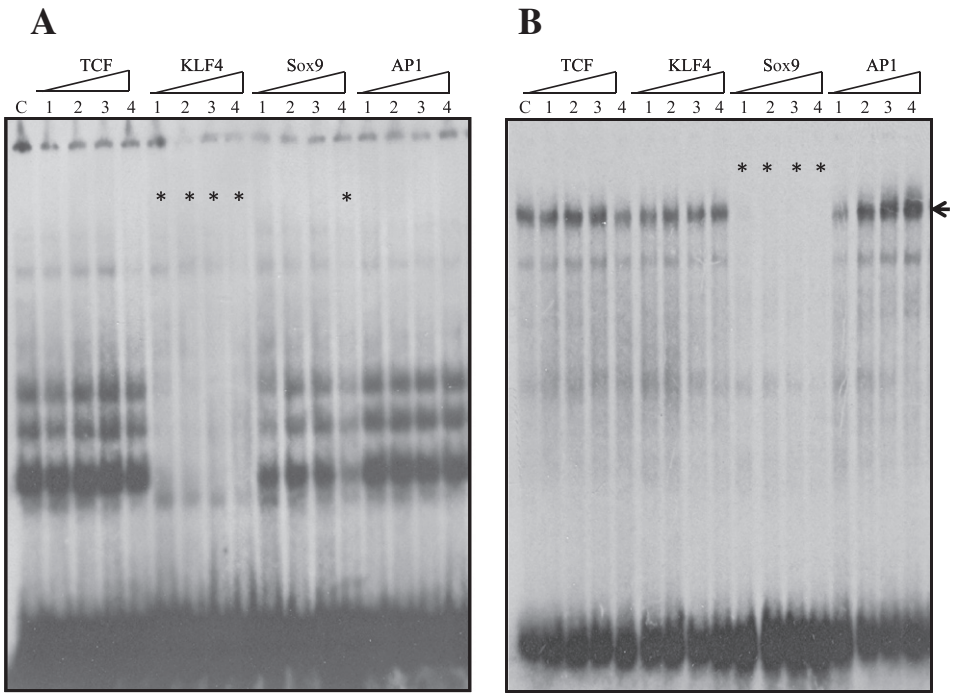


Fig. 2. TCF does not abolish Sox9 and KLF4 binding. Nuclear protein extracts were prepared from SW480 colon cancer cells and EMSA was performed as described in the Material and methods. Oligonucleotides at increasing concentration (10-, 25-, 50-, or 100-fold excess) were incubated with nuclear extract proteins (10 μg) for 30 min before adding either radiolabeled KLF4 (panel A) or Sox9 (panel B) probes and resolving proteins on native polyacrylamide gel. Figures are representative of three independent experiments.

2.3. Transfection and luciferase reporter assays

To evaluate the TCF/LEF transcriptional activity, we used a pair of luciferase reporter constructs, TOPFlash and FOPFlash (Upstate Biotechnology). TOPFlash contains three copies of the TCF/LEF binding site [AAGATCAAAGGGGGT] upstream of the thymidine kinase minimal promoter, and FOPFlash contains a mutated TCF/LEF binding site [AAGGCCAAAGGGGGT]. The underlined nucleotides were mutated in FOPFlash-luciferase reporter construct. Cells were transiently transfected with 250 ng of each luciferase reporter using Fugene HD transfection reagent (Roche, Indianapolis, IN), as instructed by the supplier. In cotransfection experiments, various concentrations of different expression vectors were combined with 250 ng of TOPFlash or FOPFlash in the presence of Fugene HD. Luciferase activities were measured 48 h post-transfection using luminometer TD20/20 (Promega). The results are expressed as a ratio of TOPFlash:FOPFlash normalized

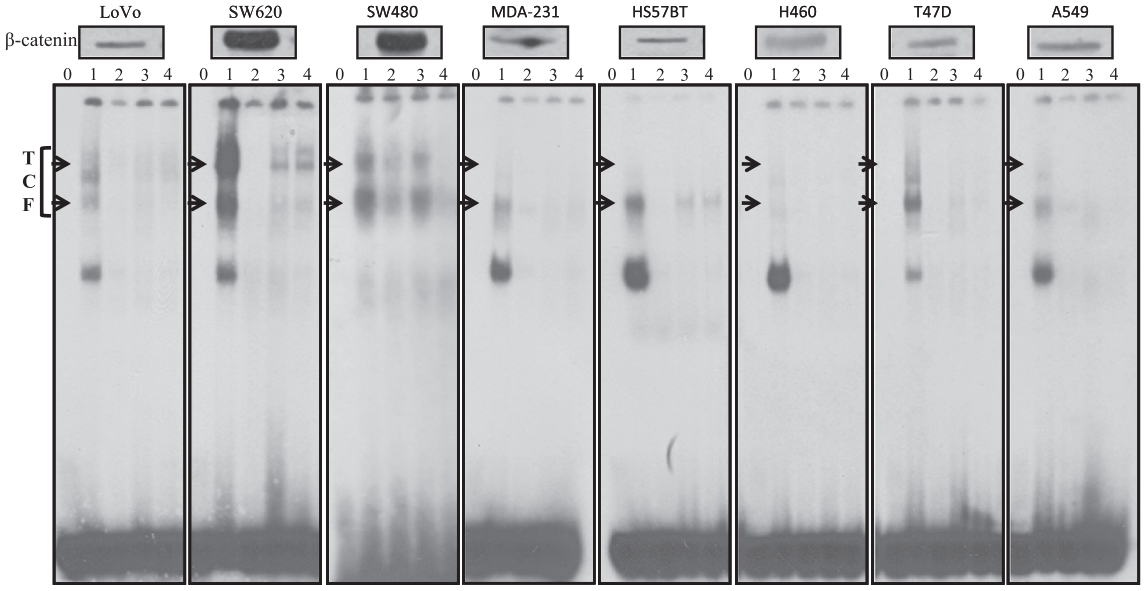


Fig. 3. Sox9 and KLF4 oligonucleotides abolish TCF-binding activity in cancer cells. Nuclear protein extracts (10 μg) were prepared from the indicated cancer cells and gel shift assays were performed as described in the Material and methods. Excess of TCF (lane 2), Sox9 (lane 3), or KLF4 (lane 4) oligonucleotides was preincubated with nuclear extract proteins (10 μg) for 30 min. After adding TCF-radiolabeled probe and additional 30 min incubation, proteins were resolved on native polyacrylamide gel. The square under the name of each cell represents a Western blot for β-catenin expression. The figure is representative of three independent experiments.

to β-galactosidase (50 ng) which served as an internal control for transfection efficiency. Expression vectors: β-catenin was from Addgene; Sox9 (sc321884) and KLF4 (sc123501) were from Origene (Rockville, MD).

2.4. Western blot analysis

Western blots were performed to verify the overexpression of proteins in control and transfected cells. Total protein extracts (60 μg) were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with anti-Sox9 (Santa-Cruz Biotechnologies, CA), anti-KLF4 (Abcam, Cambridge, MA), anti-β-catenin (Millipore, Bedford, MA), and anti-β-actin (Santa-Cruz) antibodies. The signal was detected by enhanced chemiluminescence (Pierce, Rockford, IL).

2.5. Immunoprecipitation

HEK293 cells were transiently transfected for 48 h to overexpress β-catenin/Sox9 or β-catenin/KLF4. Cells were washed twice with ice-cold PBS and extracted with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.5 mM sodium orthovanadate) containing a protease inhibitor cocktail (Pierce). Protein concentrations were determined and 200 μg of protein was incubated over night under rotation at 4 °C with antibodies (anti-KLF4 from Abcam or anti-Sox9 from Santa-Cruz) bound to protein G-agarose following the protocol provided by the company (Santa-Cruz). Immunoprecipitates were washed with RIPA buffer for five times while transferring to new tubes each time. The proteins were solubilized with reducing sample buffer and were analyzed for β-catenin expression by SDS-PAGE and immunoblotting. Immunoblots were developed using enhanced chemiluminescence (Pierce).

To demonstrate that Sox9 and KLF4 reduce the interaction between β-catenin and TCF-4, SW480 cells were transfected with different amounts of Sox9 and KLF4 (0, 0.5, or 1 μg) expression vectors for 48 h. Nuclear extracts proteins were prepared and immunoprecipitation was performed as described above using anti-TCF4 antibody (Santa-Cruz Biotechnologies) followed by Western blot using anti-β-catenin antibody. Histone H1 was used as protein loading control and detected using anti-Histone H1 antibody (Santa-Cruz Biotechnologies).

2.6. Statistical analysis

All luciferase assays were performed in at least triplicates and repeated a minimum of 3 times. Results are expressed as mean ± standard deviation (SD). Significant differences between means compared to the control were determined using Student's t-test as $^{*}p<0.05$ and $^{**}p<0.01$.

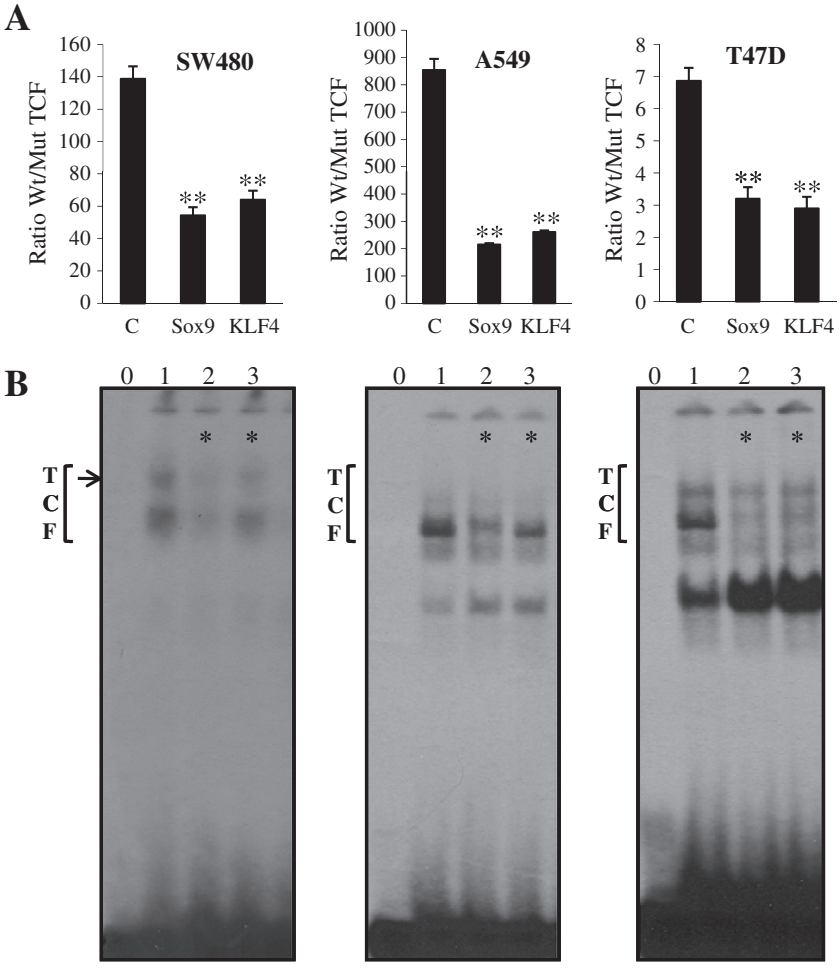


Fig. 4. Overexpression of Sox9 or KLF4 inhibits both TCF-binding and activity. Panel A, SW480, A549, and T47D cells were co-transfected with either Sox9/TCF-luciferase or KLF4/TCF-luciferase expression vectors. Forty-eight hours later, cells were lysed and luciferase assays were performed as described in the Material and methods. Figures are representative of three independent experiments performed in triplicates. $^{**}p<0.01$. Panel B, in a parallel experiment, cells were transfected with either Sox9 or KLF4 expression vectors and 48 h later nuclear extract proteins (10 μg) were prepared and incubated with TCF-radiolabeled probe for 30 min before resolving proteins on native polyacrylamide gel. 1: control; 2: Sox9; 3: KLF4 expression vectors. The figure is representative of three independent experiments.

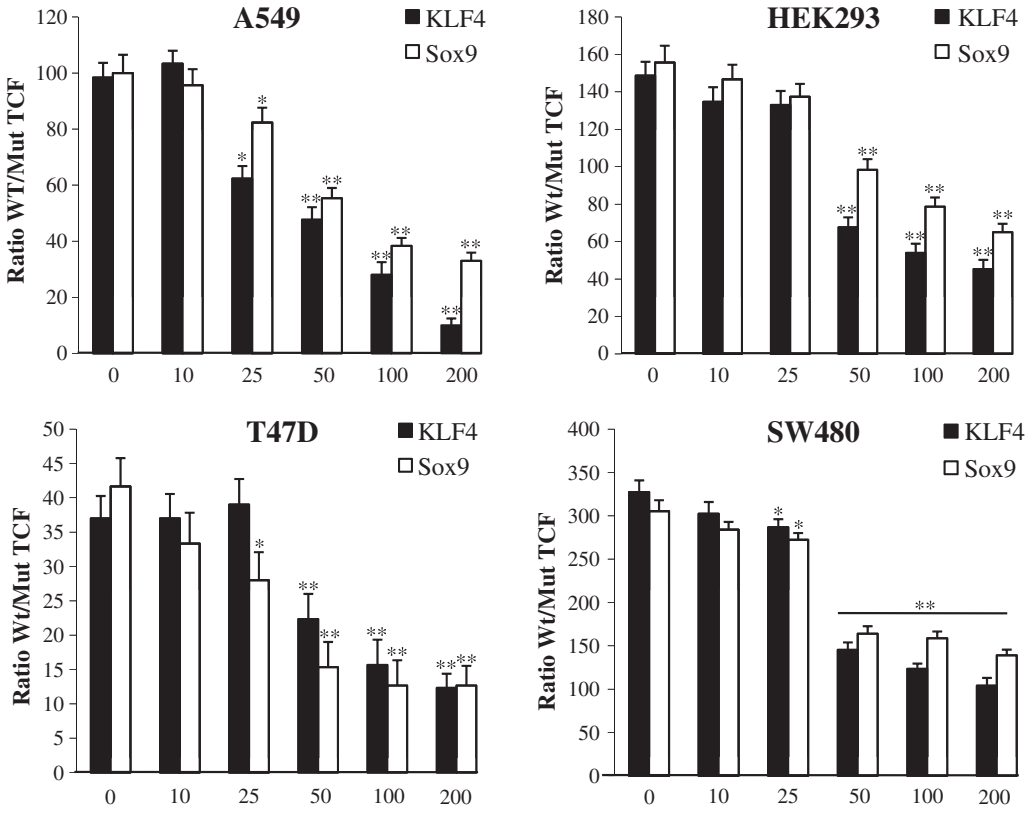


Fig. 5. Overexpression of Sox9 or KLF4 concentration dependently inhibits TCF-luciferase activity. HEK293, SW480, A549, and T47D cells were co-transfected with either Sox9/TCF-luciferase or KLF4/TCF-luciferase constructs at concentrations ranging from 0 to 200 ng. Forty eight hours later, luciferase assays were performed as described in the Material and methods. The figure is representative of four independent experiments performed in triplicates. *$p<0.05$, **$p<0.01$.

3. Results

3.1. Sox9 and KLF4 oligonucleotides antagonize β-catenin binding to TCF

In an attempt to determine which transcription factor might dislodge β-catenin binding to a synthetic TCF probe, EMSA was performed. Excess of different oligonucleotides was pre-incubated with nuclear extracts, prepared from SW480 colon cancer cells, 30 min before adding γ-^{32}P-labeled TCF probe. The sequences of consensus oligonucleotides known to bind specific transcription factors are reported in EMSA section (see Material and methods). The Sox9 consensus sequence was previously described [36] and used in this study. Fig. 1A shows the results of a representative gel shift assay to analyze β-catenin/TCF complex formation. The upper band corresponds to β-catenin/TCF shift and the lower band represents TCF binding (see arrows). We can see that TCF-binding decreased by excess of TCF (lane 2), Sox9 (lane 5), and KLF4 (lane 10) oligonucleotides. In addition to these three oligonucleotides, the sequence recognizing serum response factor SRF was also shown to inhibit TCF-binding (lane 11) but was not considered in this study. Fig. 1B represents an EMSA competition experiment using TCF as a probe and nuclear extract proteins harvested from SW480 cells. When excess of unlabeled Tcf oligonucleotide was used, all shifted bands were erased including β-catenin (upper band). Also shown in Fig. 1B the reduction of TCF binding by Sox9 and KLF4 oligonucleotides was concentration-dependent. These results suggest that the following possibilities might occur in this *in vitro* binding assay: *first*, Sox9 or KLF4 oligonucleotides, through binding to their respective transcription factors, might bind to TCF DNA and therefore oppose TCF protein binding; *second*, these oligonucleotides might compete with TCF proteins for β-catenin binding. In this situation, these oligonucleotides, through

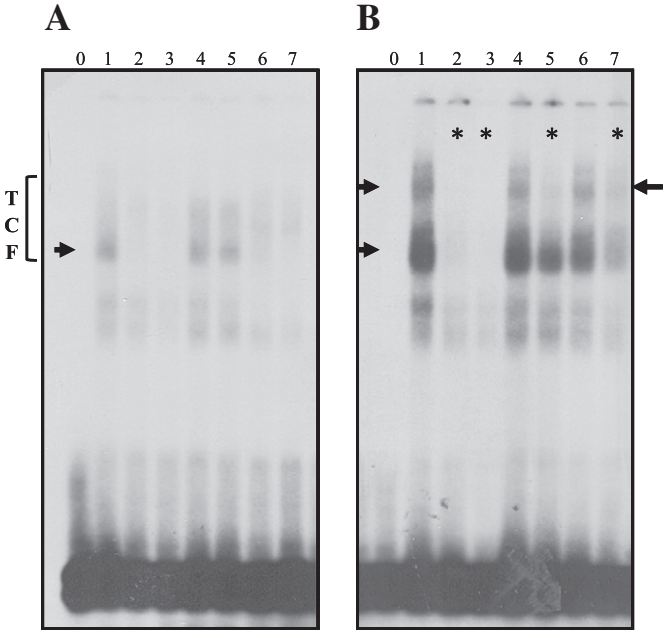


Fig. 6. Sox9 and KLF4 oligonucleotides decreased TCF-binding activity in HEK293 cells overexpressing β-catenin. Nuclear extract proteins were prepared from non-transfected HEK293 (panel A) or HEK293 cells transfected with 1 μg of β-catenin expression vector (panel B) and incubated with excess of TCF, Sox9, or KLF4 oligonucleotides for 30 min. TCF-radiolabeled probe was added and incubated for another 30 min before resolving proteins on native polyacrylamide gel. The figure is representative of at least three independent experiments.

their respective transcription factors, interact with β-catenin and oppose its binding to TCF proteins; *third*, these oligonucleotides, through binding their respective transcription factors, might recognize both β-catenin and TCF protein and ultimately inhibit TCF-binding activity. To rule out which possibility is occurring in this model, we tested whether TCF oligonucleotide could compete with either Sox9- or KLF4-radiolabeled probes.

3.2. Sox9 and KLF4 antagonize TCF binding but TCF does not abolish Sox9 or KLF4 binding

The results in Fig. 2A revealed that when KLF4 was used as a radiolabeled probe, excess of TCF oligonucleotide could not reduce KLF4 binding. However, a slight inhibition was detected when Sox9 oligonucleotide was used at a higher concentration (100-fold). Similarly, when Sox9 was used as a probe (Fig. 2B), the excess of either TCF or KLF4 oligonucleotides did not affect Sox9 binding activity. These results demonstrate that TCF oligonucleotide does not compete with Sox9 or KLF4 probes for the binding of either Sox9 or KLF4 transcription factors. This result eliminates the first possibility described above. However, the mechanism by which Sox9 and KLF4 reduce TCF-binding activity is not yet solved. To confirm whether Sox9 and KLF4 inhibition of TCF-binding activity was not mediated through competition between Sox9 or KLF4 and TCF proteins for binding to TCF DNA-sites, we examined the binding specificity of Sox9, KLF4 as well as TCF by EMSA. Gel bands obtained with TCF consensus sequence were not shifted when using antibodies directed against Sox9 or KLF4. These results suggest that neither Sox9 nor KLF4 bind to the TCF-complex (data not shown).

3.3. Sox9 and KLF4 inhibition of TCF-binding activity occurs in most tested cancer cells

To verify whether the inhibition of TCF binding by Sox9 and KLF4 excess oligonucleotides was specific to SW480 colon cancer cells, nuclear extracts were prepared from different cancer cell lines and EMSA was performed as described in the Material and methods. Results in Fig. 3 show that the TCF band (bottom arrow) is retarded in almost all the nuclear extracts prepared from different cells but with different intensities depending on the amount of TCF proteins within each cell. The shifted band corresponds to β-catenin (top arrow) as assessed by anti-β-catenin antibody. Similarly, the intensity of the shifted β-catenin/TCF intensity varies from cells to cell depending on the nuclear amount of this protein. We can see also that higher binding activities were obtained when nuclear extracts were prepared from SW620 and SW480 colon cancer cells. This strong TCF-binding activity was correlated to higher TCF-luciferase activity in these two cells (result not shown). As also shown in Fig. 3, excess of TCF (lane 1), Sox9 (lane 2), or KLF4 (lane 3) oligonucleotides inhibited TCF-binding in tested cancer cells. It is noticeable that both SW480 and SW620 shifted a band corresponding to β-catenin compared to all nuclear extracts prepared from other cells. However, longer exposure of the film showed that β-catenin was shifted in most cells (data not shown). The square under the name of each cell represents a Western blot for β-catenin expression showing that, as expected, cells express variable amounts of β-catenin. These results demonstrate that Sox9 and KLF4 oligonucleotides decrease TCF-binding in tested cancer cell lines, and suggest the plausible involvement of a common mechanism governing the interaction of these transcription factors with the β-catenin/TCF complex. However, these results do not exclude that Sox9 or KLF4 might bind both β-catenin and TCF proteins, preventing them from binding to TCF probe.

3.4. Overexpression of Sox9 and KLF4 inhibits both TCF-binding and luciferase activity

In an attempt to demonstrate whether Sox9 or KLF4 expression vectors inhibit TCF-binding activity by interacting with β-catenin, we cotransfected Sox9 or KLF4 expressing vectors with TCF-luciferase reporter constructs (TOPFlash and FOPFlash) in SW480, A549, and TD47 cancer cells and performed TCF-binding (EMSA) as well as TCF-luciferase assays. Results in Fig. 4A show that Sox9 or KLF4 cotransfection with TCF-luciferase reporter construct reduced TCF activity in SW480, A549, and T47D cells. This decrease in TCF-luciferase activity was concentration-dependent as shown in Fig. 5. In parallel, the decrease in TCF-luciferase activity was accompanied with a concomitant reduction in TCF–complex binding (see asterisk) when EMSA was performed using nuclear extracts harvested from SW480, A549 and T47D cells and TCF as a radiolabeled probe (Fig. 4B). Taken together, these data demonstrate a net correlation between the inhibition of TCF-luciferase activity and the decrease in TCF-binding following Sox9 and KLF4 transcription factors overexpression.

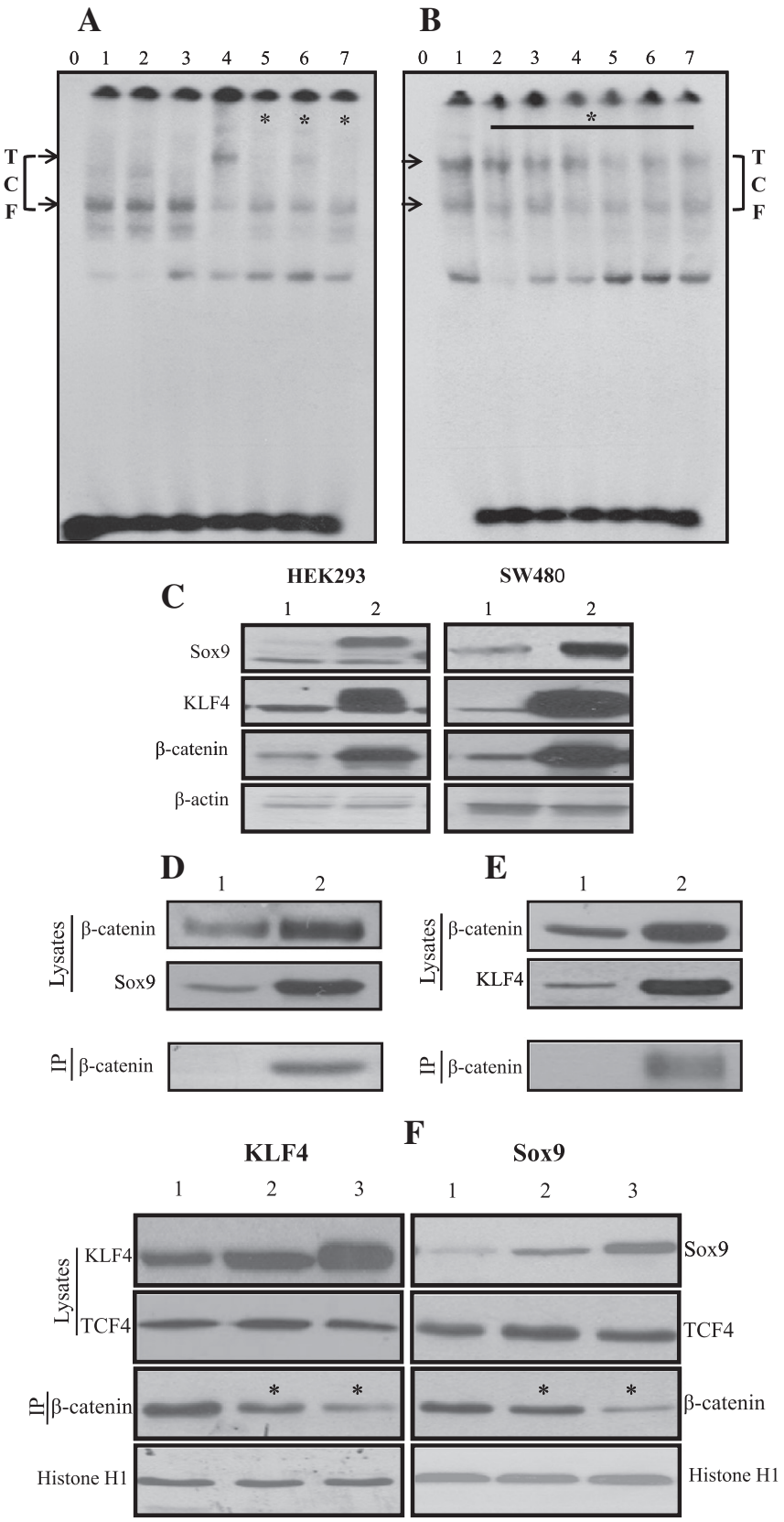
Since it has been previously reported that KLF4 can decrease β-catenin mRNA and protein levels in the human colon cancer cell line HT29 [20], we examined whether overexpression of either Sox9 or KLF4 transcription factors inhibits TCF-activity by affecting β-catenin protein levels. After overexpression of these transcription factors in SW480 cells and quantifying β-catenin levels by Western blot, we did not find any change in β-catenin expression levels (data not shown). These results suggest that Sox9 and KLF4 inhibit β-catenin binding without affecting its expression level in SW480 cells.

3.5. Sox9 and KLF4 oligonucleotides decrease TCF-binding activity in HEK293 cells overexpressing β-catenin

To address whether Sox9 and KLF4 oligonucleotides dislodge β-catenin binding to TCF complex, HEK293 cells were transiently cotransfected with β-catenin expression vector and TCF-luciferase reporter construct. Nuclear extracts were harvested 48 h post transfection, and TCF-binding assays were performed as described in the Material and methods. As shown in Fig. 6A, when β-catenin was not overexpressed (panel A), only a reduction in TCF-binding complex was obtained since no β-catenin shift was detected, even though these cells contain a detectable amount of β-catenin and express high TCF-luciferase activity, similar to SW480 and SW620 colon cancer cells (data not shown). However, when HEK293 cells were overexpressing β-catenin (Fig. 6B), β-catenin is retarded on the gel (lane 1, see arrow), and its binding was eliminated by excess of TCF (lane 2, 3), and reduced, at different intensities, by Sox9 (lane 5), and KLF4 (lane 7) oligonucleotides. These results suggest that KLF4 and Sox9 inhibit β-catenin binding to TCF proteins. However, whether KLF4 and Sox9 transcription factors could antagonize β-catenin binding in cancer cells is not yet solved.

3.6. Overexpression of Sox9 and KLF4 dislodge β-catenin binding to TCF

To demonstrate whether Sox9 and KLF4 transcription factors affect β-catenin binding, we performed EMSA using nuclear extracts prepared from HEK293 or SW480 cells overexpressing Sox9, KLF4, β-catenin, β-catenin/Sox9, β-catenin/KLF4, or β-catenin/Sox9/KLF4 expression vectors. Results in Fig. 7A show that a band corresponding to β-catenin was shifted (lane 4) in nuclear extracts from HEK293 cells overexpressing β-catenin (panel A). This band was reduced following cotransfection of β-catenin with either Sox9 (lane 5), KLF4 (lane 6) or both Sox9/KLF4 (lane 7) expression vectors. Using nuclear extract proteins prepared from SW480 (Fig. 7B), the binding was reduced by Sox9 (lane 5), KLF4 (lanes 6), and a combination of both (lane 7) compared to the control (lane 1). Results in Fig. 7C show a representative Western blot for HEK293 and SW480 cells overexpressing Sox9, KLF4, or β-catenin. These results demonstrate that Sox9 and KLF4 transcription factors prevent β-catenin from binding to TCF proteins. This inhibition is correlated to the decrease in TCF-luciferase activity shown in Fig. 4A. Taken together, these data suggest that Sox9 and KLF4 transcription factors bind to β-catenin and prevent its interaction with TCF, resulting in inhibition of both TCF-binding and TCF-luciferase activities.


A
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D
1 2
Lysates
β-catenin
Sox9
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β-catenin
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β-catenin
KLF4
IP
β-catenin
F
KLF4
Sox9
1 2 3 1 2 3
Lysates
KLF4
TCF4
IP
β-catenin
Histone H1
Sox9
TCF4
β-catenin
Histone H1

3.7. Sox9 and KLF4 interact with β-catenin

To demonstrate whether Sox9 or KLF4 transcription factors interact with β-catenin and ultimately reduce its binding to TCF proteins, HEK293 cells were transiently cotransfected with either Sox9/β-catenin or KLF4/β-catenin expression vectors. Immunoprecipitation was performed with anti-Sox9 or anti-KLF4 antibodies respectively. The blotting was performed with anti-β-catenin antibody. Fig. 7D and E shows that Sox9 and KLF4 formed a complex with β-catenin as predicted from EMSA studies. In lane 2 (bottom), results show an interaction between β-catenin and Sox9 or KLF4.

3.8. Sox9 and KLF4 inhibit the interaction of β-catenin with TCF4

We observed that Sox9 and KLF4 transcription factors reduced β-catenin/TCF binding (EMSA), inhibited TCF-luciferase activity (luciferase assays), and both interacted with β-catenin (immunoprecipitation). To demonstrate that Sox9 and KLF4 are implicated in reducing β-catenin interaction with TCF proteins, SW480 cells were transfected with these expression vectors (Sox9, KLF4), and immunoprecipitation was performed using anti-TCF4 antibody followed by immunoblot using anti-β-catenin antibody. Results in Fig. 7F show that overexpression of Sox9 (2, 3) and KLF4 (2, 3) in SW480 cells concentration dependently reduced the amount of β-catenin that immunoprecipitates with TCF4 compared to the control (1). These results demonstrate that Sox9 and KLF4 inhibit the interaction between β-catenin and TCF4.

4. Discussion

β-catenin interacts with a multitude of transcription factors that can act as a transcriptional activator, oncogene, or tumor suppressor depending on the context (cell context, expression pattern of other genes, or chromatin environment of individual cell) [38]. In most cancer cells, β-catenin, because of mutations occurring in the interacting partners, escapes the scaffolding complex where it is neutralized, and is therefore free to translocate to the nucleus where it abnormally activates TCF-related genes and compromises normal cell function [39,40]. The aim of this study was to determine which transcription factor(s) might prevent β-catenin from binding to its nuclear target, TCF proteins. For this purpose, we utilized an *in vitro* TCF-binding assay (EMSA) completed by a functional TCF-luciferase assay. Among the oligonucleotides screened, consensus sequences binding both Sox9 and KLF4 were revealed to be good candidates for inhibiting TCF-binding activity. Furthermore, these transcription factors reduced both β-catenin/TCF interaction as well as TCF-luciferase activity. Moreover, this inhibition did not result from competition between TCF and Sox9 or KLF4 DNA-binding sites. The failure of TCF oligonucleotide to inhibit Sox9 or KLF4 binding demonstrates that Sox9 and KLF4 transcription factors do not recognize and bind TCF DNA sequence, even though Sox9 binding sequence was reported to be related to TCF DNA-binding family [32,37]. Sox9 or KLF4 transcription factor overexpression in HEK293 cells induced a marked decrease in β-catenin/TCF-dependent activity of the synthetic TOPFlash luciferase sequence as well as a reduction of TCF-binding. In addition, Sox9 and KLF4 reduced the amount of β-catenin interacting with TCF proteins. These lines of evidence strongly suggest the existence of a negative functional interaction between β-catenin and Sox9 or KLF4 transcription factors and support previous reports [30].

The inconsistency in and discrepancy among KLF4 reports may reflect the pleiotropic functions of this transcription factor as well as the complexity of its contribution as a tumor suppressor [12] or oncogene [4,17,41]. It has been suggested that KLF4 modulation of β-catenin/TCF4 signaling may be context-dependent [42–44]. Our study shows that the reduction of TCF binding by Sox9 and KLF4 overexpression occurs in almost all tested cancer cells. Concomitantly, a reduction in TCF-luciferase activity was observed in analyzed cells. These results support the possibility that Sox9 and KLF4 transcription factors might have a common mechanism of action in all cancer cells. Since it was demonstrated, using GST-pull down assays and immunoprecipitation, that KLF4 binds β-catenin as well as TCF4 [4], our observations support these results and suggest that Sox9 and KLF4 transcription factors might interact with TCF proteins preventing them from binding TCF consensus sequence. In addition, it is possible that Sox9 and KLF4 perform different interactions to suppress β-catenin signaling.

Sox9 is defined as a tumor suppressor [26,27,45] or oncogene [46–48] depending on cell context and cancer type. Its effect was demonstrated to be both β-catenin-dependent and -independent [26,27,45]. However, the precise mechanism by which Sox proteins regulate β-catenin/TCF activity is not completely resolved, and few studies suggest the involvement of different mechanisms including protein–protein interaction, the binding of Sox factors to TCF-target gene promoters, the recruitment of co-repressors or co-activators, and the regulation of protein stability [30]. A negative interaction between Sox9 and β-catenin has been previously documented during chondrocyte differentiation in which dimerization of Sox9 and β-catenin leads to the mutual degradation of both proteins [30]. In addition, the existence of an antagonism between Sox9 and β-catenin has been reported [49]. Thus, it is likely that Sox9 binds to β-catenin and excludes TCF proteins from interacting with it. This inhibitory model of Sox9 is a feature common to KLF4 as well. They both interact with β-catenin and prevent it from binding to TCF. However, it is not excluded that Sox9 and KLF4 bind to TCF proteins and prevent them from binding to TCF DNA sequence as well as to β-catenin.

Sox9 and KLF4 could suppress β-catenin/TCF activity by mediating degradation of either β-catenin or TCF proteins. However, following our *in vitro* cotransfection experiments, neither Sox9 nor KLF4 could cause β-catenin degradation, supporting the observation of Zhang et al. [19] where KLF4 reduces TCF-activity but does not affect neither wild type nor mutated β-catenin levels. This possibility does not occur in our model contrary to what was previously reported [18,29,31] and where KLF4 downregulates the level of β-catenin by direct binding and inhibition of its transcriptional activation domain. In addition, a recent report shows that the decrease in β-catenin/TCF transcriptional activity was not due to reduced interaction between β-catenin and TCF4 but was associated with an increased interaction between β-catenin and KLF4.

As Sox and TCF proteins bind similar DNA sequences, Sox proteins might also suppress Wnt-induced transcription by competing

Fig. 7. Panels A and B. Overexpression of KLF4 and Sox9 decreased β-catenin binding to TCF. HEK293 (panel A) or SW480 (panel B) cells were transfected with Sox9 (lane 2), KLF4 (lane 3), β-catenin (lane 4), β-catenin/Sox9 (lane 5), β-catenin/KLF4 (lane 6), or β-catenin/Sox9/KLF4 (lane 7) expression vectors (1 μg each). Nuclear extract proteins were prepared 48 h post transfection and gel shift assays were performed as described in the Material and methods using TCF as a radiolabeled probe. Lane 1 corresponds to control. Panel C. Western blot showing the overexpression (2) of Sox9, KLF4, and β-catenin in HEK283 and SW480 cells compared to non-transfected cells (1). Panels D and E. Sox9 and KLF4 interact with β-catenin. HEK293 cells were transiently cotransfected (2) for 48 h with Sox9, β-catenin, or KLF4 expression vectors (1 μg each). Immunoprecipitation (IP) was performed 48 h later with either Sox9 (Panel D) or KLF4 (Panel E) antibodies. After running the precipitate on 10% SDS-PAGE gel and transfer, nitrocellulose membranes were analyzed with anti-β-catenin antibody. Panel F. Sox9 and KLF4 reduce the interaction of β-catenin with TCF4. SW480 cells were transfected with Sox9 or KLF4 expression vectors at different amounts (1: 0 μg, 2: 1 μg, 3: 2 μg) for 48 h. Nuclear extracts were prepared as mentioned in the Material and methods, Immunoprecipitation was performed using anti-TCF4 antibody (Santa-Cruz) followed with immunoblot using anti-β-catenin antibody. Results in Fig. 7F show that overexpression of Sox9 or KLF4 in SW480 results in a decrease of the amount of β-catenin interacting with TCF. Histone H1 was used as a loading control for nuclear extract proteins using anti-Histone H1 antibody (Santa Cruz). The figure is representative of three independent experiments.

with TCF for the same promoter sites. Our *in vitro* gel shift assays showed that Sox9 could inhibit TCF binding but TCF did not affect Sox9 or KLF4 binding activities. In addition, *in vitro* DNA-binding studies with optimized Sox and TCF DNA-binding sites argue against this model, and conclude that Sox proteins bind very poorly, if at all, to an optimized TCF consensus sequence and vice versa [33,35]. Subsequently, many other Sox proteins from a variety of species have been shown to repress β-catenin stimulated TOPFlash transcription in tissue culture as well as complexing β-catenin [19,24,30,34,35].

In conclusion, our results demonstrate that Sox9 and KLF4 are binding to β-catenin and reducing its interaction with TCF proteins. As a consequence, these transcription factors inhibit TCF activity and ultimately TCF-dependent genes activation. Given the critical role of β-catenin in mediating Wnt signaling as well as in the development of cancer, the identification of inhibiting or neutralizing partners will lead for a novel therapy for cancer.

Abbreviations

TCF/LEF	T-cell factor/lymphoid-enhancing factor
EMSA	Electrophoretic mobility shift assay
KLF	Krüppel-like factor
BSA	bovine serum albumin

Conflict of interest

No potential conflict of interest was disclosed.

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