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The balance of TCF7L2 variants with differential activities in Wnt-signaling is regulated by lithium in a GSK3 β -independent manner

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Abstract

TCF7L2 transcription factor is a downstream effector of the canonical Wnt/ β -catenin signaling, which controls cell fate and homeostasis. However, the complexity of *TCF7L2* expression with numerous mRNA isoforms coding for proteins with distinct N- and C-termini allows variability in TCF7L2 functions and regulations. Here, we show that although TCF7L2 mRNA isoforms distinguish fetal, immortalized and adult differentiated endothelial cells (EC), they cannot explain the lack of significant β -catenin/TCF7 activities in ECs. Lithium, a Wnt-signaling activator, increases TCF7L2 mRNA levels and induces an RNA isoform switch favoring the expression of TCF7L2-short forms lacking the C-termini domains. Although the latter occurs in different cell types, its extent depends on the overall increase of *TCF7L2* transcription, which correlates with cell-responsiveness to Wnt/ β -catenin signaling. While GSK3 β down-regulation increases *TCF7L2* expression, there is no concomitant change in TCF7L2 mRNA isoforms, which demonstrate the dual effects of lithium on *TCF7L2* expression via a GSK3 β -dependent up-regulation and a GSK3 β -independent modulation of RNA-splicing. TCF7L2E-long forms display a repressor activity on *TCF7L2*-promoter reporters and lithium induces a decrease of the endogenous TCF7L2 forms bound to native *TCF7L2*-promoter chromatin at two novel distal TCF7-binding sites. Altogether our data reveal a lithium-induced RNA switch favoring the expression of TCF7L2-short forms, which results in a transcriptional de-repression of lithium-target genes negatively regulated by TCF7L2-long forms, like *TCF7L2*, and thus to an amplification of Wnt-signaling in responsive cells.

Keywords

β -catenin; alternative-splicing; endothelial cells; immortalization

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Introduction

Canonical Wnt/ β -catenin signaling is involved in the control of cell fate during development and in stem cell renewal in the adult [1]. In this pathway, inhibition of β -catenin phosphorylation by GSK3 β triggers β -catenin stabilization and its accumulation in the cytoplasm and subsequently in the nucleus where β -catenin acts as co-activator for the TCF7/LEF1 transcription factors. TCF7/LEF-1 factors belong to a small High-Mobility-Group (HMG) Box subfamily comprising 4 members: TCF7/TCF1, TCF7L1/TCF3, TCF7L2/TCF4 and LEF-1. Although they all bind the consensus A/TT/ACAAAG DNA sequence and share similar protein-protein interaction domains such as the β -catenin-binding domain and interacting domain for the co-repressors Groucho/TEL, they display differential activities regarding regulation of β -catenin/TCF7 target genes in different tissues and cell types [2].

Functional TCF7L2 diversity is also achieved by alternative RNA-splicing and exon-skipping that result in the expression of TCF7L2 forms with different N- and C-termini and thus variability in interacting partners and regulatory networks in a cell-context and target-gene dependent manner [3,4]. The variable domains are, in the C-terminus, two binding sites for the co-repressor CtBP and a C-clamp involved in DNA-binding specificity and affinity, and in the N-terminus, the β -catenin binding domain and small intervening sequences between the β -catenin- and Groucho-binding domains, which may regulate these TCF7L2-interactions [2,4–6].

While activation of the Wnt/ β -catenin signaling, via mutations of β -catenin and several Wnt-signaling components, is highly associated with the development and progression of colon- and hepato-carcinoma [1], only rare mutations of *TCF7L2* have been reported. Instead, specific TCF7L2-RNA isoforms were found enriched in colon carcinoma [3,5]. Common polymorphisms distributed all along *TCF7L2* gene have been associated with increased risks to develop Type-2 diabetes [7,8] and its associated cardiovascular complications [9]. In this setting also, differential TCF7L2 mRNA levels and specific TCF7L2 mRNA isoforms are thought to depend upon *TCF7L2* genotypes [10,11].

Lithium inhibits GSK3 β and thereby activates β -catenin/TCF7 transcriptional activities in different immortalized and cancer cells [12]. However, we have previously shown that lithium does not induce β -catenin/TCF7 transcriptional activities in primary ECs, but induces rather a cell cycle arrest and activation of the tumor suppressor p53 leading subsequently to the establishment of cell senescence [13,14]. Similarly, expression of stable β -catenin forms leads to development of features associated with cell senescence *in vitro* and tissue aging *in vivo* [15,16]. Sustained activation of the Wnt3a/ β -catenin signaling pathway in the skin has also been associated with premature tissue aging and increased TCF7-dependent activities were detected in aging skeletal muscles [17,18]. Since *TCF7L2* itself is a β -catenin target gene [19], we tested the hypothesis that specific expression of TCF7L2 variants in a cell context-dependent manner mirrors the responsiveness to and/or the outcome of Wnt/ β -catenin signaling activation by comparing the expression of TCF7L2 forms and their Wnt-signaling-dependent regulations in ECs and in the responsive stem-like HEK293 cells.

Material and Methods

Materials

LiCl was purchased from Sigma-Aldrich and all the primary and secondary antibodies were from Cell Signaling. The oligonucleotide primers were synthesized by Integrated DNA Technologies and the sequences are reported in the Supplementary Table S1.

Mammalian expression constructs

The S37A- β -catenin-HA tagged construct was previously described [20]. The full-length cDNAs for the TCF7L2 variants expressed in ECs were amplified by RT-PCR from HUVEC RNAs, N-terminus HA-tagged and cloned into pCR3.1-TA vector (Invitrogen).

Cell culture and transfection

Primary BAEC (Cambrex) were maintained in DMEM containing 1g/L D-glucose, 5% FBS and 100U/ml penicillin-100 μ g/ml streptomycin (Gibco-Invitrogen). HEK293T (Invitrogen) and the retinal RF6A (ATCC) cells were maintained in DMEM containing 4.5g/L D-glucose, 10% FBS and antibiotics. DNA transfections were performed using the Exgen500 reagent (Fermentas).

Short-interfering RNA transfection

The human/bovine siRNA-GSK3 β duplex (5'-ACACUAUAGUCGAGCCAAUu-3') was previously described [14]. The on-target-plus Non-targeting-siRNA#1 was used as control and the si-RNA transfections were achieved using Dharmafect-I reagent (Dharmacon Inc.).

RNA extraction, reverse transcription and real-time PCR

RNA extractions were performed using Trizol-reagent (Invitrogen). Total RNAs (1 μ g) were subjected to DNase-I treatment and reverse-transcription prior to PCRs with an equivalent of 10ng RNA per reaction. Quantitative real-time PCRs were performed in duplicates using SyBr-Green core reagents and the ABI7000/ABI7900 apparatus (Applied-Biosystems) as previously described [21] and normalized with the levels of rpL30 mRNAs.

Reporter assays

Cells were transiently co-transfected in duplicates with 5ng pCMV- β -galactosidase for normalization, 125ng of the luciferase-reporters and 400ng of the S37A- β -catenin-HA or HA-TCF7L2 variants. The luciferase reporters used in this study are i) the TOP-/FOP-Flash and the OT/OF reporters that contain either wildtype (TOP/OT) or mutated (FOP/OF) TCF7-binding sites either directly in front of the luciferase-cDNA (OT/OF) or upstream of the minimal thymidine kinase-promoter (TOP/FOP) [22] and ii) two TCF7-dependent promoter-luciferase constructs, the -6150/+476-HuTCF7L2P [19,23] and the 1G-2235/+60-MMP1P [14,24]. The cells were lysed 36h post-transfection and the firefly luciferase and β -galactosidase activities were quantified using Luciferase assays (Promega) and Galacto Light Plus assays (Applied Biosystems) respectively and an LMax-II luminometer (Molecular Devices). The activities of firefly luciferase were normalized with those of β -galactosidase.

Whole cell extracts and Western Blot analysis

After cold PBS washes, the cells were lysed in 50mM HEPES pH7.4, 150mM NaCl, 1% Triton-X100 supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Equal amount of proteins were fractionated on SDS-polyacrylamide gels and transferred onto Immobilon-P membrane (Millipore). After incubation with primary and secondary HRP-conjugated antibodies, the immuno-reactive proteins were detected using SuperSignal® chemiluminescence (Pierce Chemical Co).

Chromatin Immunoprecipitation (ChIp) assays

Cells were treated with either 10mM NaCl or 10mM LiCl for 36h prior to cell harvesting and native chromatin isolation. Chromatin-DNA fragments were obtained by micrococcal-DNase-I digestion (Enzymatic ChIp-assay, Cell Signaling) and 100 μ g of chromatin/DNA

were incubated with non-immune-Ig (negative control), anti-Histone-H3 antibodies (positive control) or anti-TCF7L2 antibodies for 16h at 4°C. The immune complexes were pulled down in presence of protein-G-magnetic beads and the associated DNA fragments were purified and identified by PCR using primer sets encompassing consensus TCF7-binding sites within the distal *hu-TCF7L2* promoter (Supplementary Table S1).

Statistical analysis

Student's t-tests were performed using GraphPad Prism version 4.0 (GraphPad Software) and results were considered significant at $p < 0.05$.

Results and Discussion

Increased expression of endothelial-TCF7L2 variants cannot reverse the lack of significant β -catenin/TCF7 transcriptional activity in EC

Unlike HEK293 cells, ECs of different origins do not display significant β -catenin/TCF7 transcriptional activity in response to lithium [13] and stable forms of β -catenin (Fig. 1B). To determine whether differential *TCF7L2* expression was responsible for this lack of β -catenin/TCF7 activity, we characterized TCF7L2 isoform expression in primary and immortalized ECs by performing RT-PCR with primer sets encompassing the alternative exons-4/6 and exons-13–16 (Fig. 1B) and by cloning the full-length TCF7L2 cDNAs expressed in HUVEC (Fig. 1A). The four major TCF7L2 splice variants expressed in HUVEC lack the exon-16, which is in agreement with its reported tissue-restricted expression [4,10]. HUVEC express the long-TCF7L2E form containing the CtBP-binding sites and C-clamp, and the short-TCF7L2B form lacking both domains (Fig. 1A). HUVEC express also TCF7L2C and TCF7L2D variants that display the same C-termini coded by the association of Exon-14 with an A(10) stretch in Exon-17 [3], and thus contain the CtBP-binding sites but only an incomplete C-clamp [2,4]. TCF7L2D contains also the Exon-4-coded domain that may regulate TCF7L2 interactions with β -catenin and/or Groucho.

This distribution of TCF7L2 variants in HUVEC can also be followed by PCRs using primer sets encompassing Exons-3–9 and Exons-12–17 (Fig. 1B), where the relative abundances of TCF7L2 mRNA isoforms containing Exon-4 (low) and Exon-14 (high) are consistent with the cloning data. Exon-15 and Exon-16, which have the same length in most species, cannot be distinguished on agarose and acrylamide gels (Figs. 1B–2B), but based on our cloning data the bands labeled Exon15/16 contain in majority Exon-15. The expression of Exons-4/6 and Exon-14 vary among the ECs studied. RF6A and HUVEC, which have embryonic origins, present the lowest relative levels of Exon-4-containing forms like the embryonic stem-like HEK293 cells (Fig. 1B). ECs derived from adult tissues display significant expression of the Exon-4 though it is more prominent in the primary BAEC than in the immortalized HMEC1 [25] and Br-MVEC1 [26]. The immortalization processes induced by viral T-antigens include senescence by-pass and acquisition of stem-like features [27], and thus the decreased expression of TCF7L2-Exon-4 in the immortalized adult ECs seems to reflect those phenotypic changes rather than differences between macro- and micro-vascular ECs. In contrast, the expression of Exons-13,14 RNA forms seems to differentiate macro- and micro-vascular ECs, since the lowest relative levels are found in BAEC, which instead express higher levels of the Exons-13,15 and Exons-13,14,15 RNA forms (Fig. 1B). As expected, these differential expressions of TCF7L2 RNA isoforms translate into differential expressions of TCF7L2-long and -short forms between BAEC and RF6A that can be visualized by western blotting (see Fig. 2C).

Although all the major TCF7L2 variants expressed in ECs contain the N-terminal β -catenin-binding domain, they failed to increase β -catenin/TCF7 activity in BAEC when co-

expressed with the stable S37A- β -catenin form (Fig. 1C). The short-TCF7L2B forms increased the activity of both FOP- and TOP-Flash reporters indicating general effects on transcription rather than TCF7-dependent effects (Fig. 1C). In contrast, the expression of the TCF7L2E variant decreased the activities of both FOP- and TOP-Flash reporters resulting in a 40% decreased TOP/FOP ratio, while TCF7L2D had no effect (Fig 1C). A similar TCF7L2E-mediated decrease of the basal TOP/FOP ratio was also observed in HEK293 (see Fig. 3A) and in RF6A (not shown). These data are consistent with the increased DNA binding affinity and transcriptional activity conferred by the C-clamp [4,6] and pinpoint a repressor role for TCF7L2E-long forms on this TOP/FOP system. Nevertheless, the expression of specific TCF7L2 isoforms is insufficient to explain the poor β -catenin/TCF7 activity in BAEC and RF6A since they express predominantly the transcriptional active TCF7L2-long forms and the “permissive” TCF7L2-short forms respectively.

Lithium increases TCF7L2 mRNA expression and affects TCF7L2 mRNA isoform patterns to favor the expression of TCF7L2-short forms

Since increased β -catenin/TCF7 activity can occur in part through TCF7L2 up-regulation, such as in response to vitamin-D in Wnt/ β -catenin responsive cells [23], we investigated whether TCF7L2 expression was differentially regulated upon cell responsiveness to Wnt/ β -catenin signaling by assessing the mRNA and protein levels of TCF7L2 isoforms after lithium treatment in BAEC and RF6A as well as in the Wnt/ β -catenin responsive HEK293 cells. As shown in Fig. 2A, lithium treatment resulted in a significant 2.4 fold increase of total TCF7L2 mRNAs in HEK293 cells, while there was a trend for an increase in BAEC (1.4 fold) and a slight decrease in RF6A cells (Fig. 2A). TCF7L1 mRNA expression was similarly regulated by lithium though to a lesser extent in BAEC and HEK293 cells (Fig. 2A). In addition to increase TCF7L2 mRNA expression in HEK293 cells, lithium induced also drastic changes in the patterns of TCF7L2 mRNA isoforms containing the alternative Exons-13–16. An overall 50% decrease of the mRNA forms containing Exons-13,14,15/16, Exons-13,15/16 and Exons-13,14 was observed, while the mRNA forms containing only Exon-13 increased ~ 1.5 fold (Fig. 2B). This led to the concomitant decrease of the TCF7L2-long forms and increase of the TCF7L2-short forms as detected by western blotting (Fig. 2C), and thus to an inversion of the ratio TCF7L2-long/-short forms (3 to 0.25). Although lithium treatment induced similar TCF7L2 RNA isoform switch in BAEC (Fig. 2B) its limited extent, which follows the smaller increase of total TCF7L2 mRNA levels (Fig. 2A), failed to reverse the TCF7L2-long/-short form ratio (3 to 1.8) (Fig. 2C). A slight increase of the TCF7L2-short forms was also detected in lithium-treated RF6A though without significant effect on TCF7L2 form balance since it was already in favor of TCF7L2-short forms (0.3 to 0.25) (Figs. 2B–C). In all the cells, acute lithium treatments had no significant effect on the expression of TCF7L2-RNA isoforms containing the Exons-4/6 (Fig. 2B). The concomitant decrease of the longer TCF7L2-RNA forms (Exons-13,14,15; Exons-13,15; Exons-13,14) and increase of the shorter TCF7L2-Exons-13 RNA form argue for an effect of lithium on TCF7L2-RNA splicing and more precisely on exon-skipping rather than on the stability of specific TCF7L2-RNA isoforms. Thus, lithium affects TCF7L2 mRNA isoform pattern in a rather specific fashion and its extent in different cells seems to correlate with the strength of β -catenin/TCF7 activities.

GSK3 β down-regulation and β -catenin-stabilization increase TCF7L2 mRNA expression without affecting TCF7L2-RNA isoform patterns

To determine the involvement of lithium-induced GSK3 β inhibition on TCF7L2-RNA expression and patterns, GSK3 β down-regulation by specific si-RNA duplexes was undertaken as previously described [14]. Down-regulation of GSK3 β levels by 60–50% in HEK293 cells resulted in 2.2 and 1.4 fold increase of TCF7L2 and TCF7L1 mRNA levels respectively (Fig. 3A). Lithium treatment did not significantly increase further both TCF7L2

and TCF7L1 mRNA levels (Fig. 3A), indicating that lithium-induced *TCF7L2* up-regulation is GSK3 β -dependent. However, GSK3 β down-regulation did not affect the distribution of TCF7L2 mRNA isoforms containing the alternative Exons-13–16 as well as the lithium-induced TCF7L2-RNA isoform switch favoring TCF7L2-short forms (Fig. 3A). Similarly, the ectopic expression of the stable S37A- β -catenin form increases the levels of both TCF7L2-long and -short forms but had no significant effect on the balance between TCF7L2 variants (Fig. 3B). These data are thus consistent with two inter-related but independent lithium-induced events: a GSK3 β -dependent increase of *TCF7L2* transcription and a GSK3 β -independent effect on TCF7L2-RNA splicing. The former event is in agreement with *TCF7L2* being itself a Wnt/ β -catenin target gene [19]. Since TCF7L2 variants have clearly differential transcriptional activities (Fig. 1C, [4,5]), the latter represents a novel lithium-dependent modulation of Wnt-signaling which distinguishes Wnt/ β -catenin signaling responsive and unresponsive cells.

Lithium-induced TCF7L2 isoform switch is accompanied by a decrease of distal TCF7L2-promoter occupancy by TCF7L2 in HEK293 cells

To determine the consequences of lithium-induced TCF7L2 isoform switch, we assessed the transcriptional activities of TCF7L2 variants on different TCF7-dependent reporters: the synthetic TOP/FOP and OT/OF reporter systems that differ by the absence of minimal promoter sequences in the latter and two promoter-luciferase reporters, -6150/+476-*huTCF7L2P*-pGL3 and 1G-2235/+60-*MMP1P*-pGL3 [14,23], corresponding to two lithium-target genes, *TCF7L2* (Fig. 2A) and *MMP-1* [14], containing consensus TCF7-binding sites in their proximal promoters [19,24]. In HEK293 cells, the TCF7L2E variant acted as a repressor on the TOP/FOP system (50% decrease) and as an activator on the OT/OF system (3 fold increase) (Fig. 4A), which further confirms the dual transcriptional activities of TCF7L2E depending on the neighboring promoter sequences [2,4]. TCF7L2D displayed a weaker repressor activity (20% decrease) but no activator role, while the short-TCF7L2B variant had no activity (Fig. 4A). In contrast to its expected positive effects on *MMP1*-promoter reporter [24], TCF7L2E repressed unexpectedly *TCF7L2*-promoter activity by 50% (Fig. 4A). Since all the activities were tested in the absence of β -catenin overexpression, they likely represent the basal TCF7L2 activities on the corresponding promoters in HEK293 cells. Compared to the increase of TCF7L2 (2.4 fold) and MMP1 (6 fold) RNA levels observed in response to lithium (Fig. 2A and [14]), there were only weak increases of the corresponding promoter activities: 1.2 and 1.7 respectively (Fig. 4A). Nonetheless, lithium had the opposite effects of TCF7L2E on the -6150-*huTCF7L2*-promoter reporter as well as on the -2068-*muTcf7l2*-promoter reporter [23] (not shown), suggesting that, instead of a TCF7L2-dependent activation, a decrease of TCF7L2E-repressor activity on *TCF7L2*-promoter may occur in response to lithium via its effect on the balance between active and permissive TCF7L2 variants.

Next, we analyzed the changes in the *huTCF7L2*-promoter occupancy by endogenous TCF7L2 in response to lithium using ChIP assays. First, in addition to the proximal +80 site previously described [19], two putative TCF7-binding sites located at -1813 and -2468 were found highly conserved between species. Both -1813 and -2468 distal sites showed specific binding of endogenous TCF7L2 by ChIP assays (Fig. 4B) and thus represent novel functional TCF7-binding sites within *TCF7L2*-promoter. After lithium treatment, a significant decrease of TCF7L2-bound to the more distal -2468 site was observed as well as to a lesser extent to the -1813 site (Fig. 4B), indicating that lithium affects negatively *TCF7L2*-promoter occupancy by TCF7L2 forms. Altogether these data, based on the drastic differences in transcriptional activity of TCF7L2 variants, are thus consistent with a basal repression of *TCF7L2*-promoter activity through binding of the TCF7L2E variants to the distal -2468 and -1813 TCF7-binding sites, which is released by the lithium-induced switch

to the permissive TCF7L2-short forms. Beside an intrinsic decreased DNA-binding activity, it is likely that changes in interactions with neighboring partners participate also in the differential binding of TCF7L2-short form onto *TCF7L2*-promoter.

In summary this study reports novel effects of lithium on Wnt/ β -catenin signaling via dual modulations of *TCF7L2* expression. Lithium increases TCF7L2 mRNA levels in a GSK3 β -dependent manner and induces a GSK3 β -independent RNA-isoform switch favoring the expression of TCF7L2-short forms. The extent of the latter mirrors the ability of lithium to increase β -catenin/TCF7 activities. Our data confirm the differential transcriptional activities of TCF7L2 variants and emphasize the ability of TCF7L2E to act either as an activator or repressor depending on the promoter-context [2,4]. Furthermore, we identified the repression of *TCF7L2*-promoter activity by TCF7L2E and showed that lithium-induced increase of TCF7L2-short forms is accompanied with a decrease of TCF7L2-bound onto *TCF7L2*-promoter native chromatin indicative of a de-repression mechanism of *TCF7L2* transcription rather than TCF7-dependent activation *per se*. Such de-repression of TCF7-target genes may participate in an amplification of Wnt/ β -catenin signaling, which further distinguish responsive and unresponsive cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Expression and activity of TCF7L2 isoforms in ECs. (A) Schematic representation of human *TCF7L2* exon organization [3,4], of TCF7L2 domains and of the major TCF7L2 forms expressed in HUVEC. The primer sets used to analyze the expression of total TCF7L2 mRNA (T) and the alternative exons-4/6 (Ex3–9) and exons-13/16 (Ex12–17) are represented. (B) RT-PCR analysis of the TCF7L2 mRNA isoforms expressed in ECs with different tissue origins and immortalization status. The PCR products were resolved on 1% agarose gels. (C) Analysis of the transcriptional β-catenin/TCF7L2 isoforms activity in BAEC using co-transfection of S37A-β-catenin with TCF7L2 variants as indicated and TOP/FOP-Flash reporter assays (Mean±SEM, n=4–5). A representative western-blot analysis of the endogenous and exogenous TCF7L2 isoforms is shown.

Figure 2.

Lithium increases TCF7L2 mRNA expression and favors the expression of TCF7L2 RNA isoforms coding for TCF7L2-short forms. Cells were treated with either 10mM NaCl or 10mM LiCl for 36h prior to RNA isolation and analysis of total TCF7L2 RNA levels by real-time PCR (Mean $\pm$ SEM, n=4–5, *p<0.05) (A), of TCF7L2 isoforms (Exons-3–9, Exons-12–17) by standard PCR and 8%-acrylamide gel electrophoresis (B), and prior to whole-cell lysis and analysis of TCF7L2 forms by western blotting (C).

Figure 3.

Down-regulation of GSK3 β and expression of stable β -catenin forms affect TCF7L2 levels but not the balance of TCF7L2 variants. (A) Analysis of TCF7L2 RNA levels (Mean $\pm$ SEM, n=3, *p<0.05), RNA isoform patterns and protein variants in HEK293 cells transfected with either si-control#1 (Ct) or si-GSK3 β duplexes and treated with either 10mM NaCl or 10 mM LiCl. (B) Analysis of TCF7L2 isoform expression by western blotting in HEK293 cells transfected with either PCR3-control or S37A- β -catenin constructs and treated 16h later with either 10mM NaCl or 10mM LiCl for an additional 24h period.