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e2f1 gene is a new member of Wnt/β-catenin/Tcf-regulated genes

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ABSTRACT

HDAC inhibitors induce cell cycle arrest of E1A + Ras-transformed cells accompanied by *e2f1* gene down-regulation and activation of Wnt pathway. Here we show that *e2f1* expression is regulated through the Wnt/Tcf-pathway: *e2f1* promoter activity is inhibited by sodium butyrate (NaB) and by overexpression of β-catenin/Tcf. The *e2f1* promoter was found to contain two putative Tcf-binding elements: the proximal one competes well with canonical Tcf element in DNA-binding assay. Being inserted into luciferase reporter vector, the identified element provides positive transcriptional regulation in response to β-catenin/Tcf co-transfection and NaB treatment. Thus we have firstly demonstrated that *e2f1* belongs to genes regulated through Wnt/β-catenin/Tcf pathway.

Introduction

Wnt/β-catenin signaling pathway plays an important role in processes of cell proliferation, differentiation and malignant transformation [1]. β-Catenin, a key component of the Wnt pathway, participates in two polar functions: (a) in complexes with transmembrane proteins of cadherin family it is involved in organization of cell–cell contacts and cell adhesion, (b) upon activation of Wnt-signaling β-catenin acquires a capability to move into the nucleus where in complexes with Lef/Tcf transcription factors it transactivates Wnt-regulated genes [2]. Among Wnt-regulated genes which have Tcf-responsive elements in their promoters, there are many those involved in cell cycle progression, cytoskeleton organization and differentiation: *c-myc*, *cyclin D1*, *Pitx2*, *ID2*, *nucleophosmin*, *vimentin*, *TLE/Groucho*, *REST/NRSF*, *CBP/p300*, *Frizzled* [3,4]. Transcription factors of Lef/Tcf family (Tcf 1–4) are considered as repressors, but in combination with co-activators such as β-catenin, they are capable of transactivating gene transcription. In dependence on the promoter context, β-catenin can form complexes with various regulatory proteins thereby providing positive or negative regulation of target genes [5,6].

HDAC inhibitors (HDACi) are widely used as antiproliferative agents, which are able to induce cell cycle arrest, apoptosis or senescence in various cultured tumor cell lines. A number of HDAC inhibitors are currently recommended for clinical using to cure certain blood malignancies and solid tumors [7]. The mechanism of antiproliferative effect of HDAC inhibitors is based on the suppression of histone deacetylase activity. HDACi up-regulate transcriptionally inactive negative regulators of cell cycle (*p21/Waf1*, *gadd45*, etc.) or down-regulate transcription of proliferation-promoting genes such as *c-myc*, *cyclin D1*. Rodent embryo fibroblasts transformed with oncogenes E1A and cHa-ras fail to undergo cell cycle arrest in response to DNA damage and stress factors due to disfunction of p53, p21/Waf1 and the lack of checkpoint control [8]. Nevertheless, in E1A + Ras-transformed cells HDAC inhibitors sodium butyrate and TSA induce irreversible cell cycle arrest and senescence concomitantly with down-regulation of *e2f1* and up-regulation of *β-catenin* gene transcription [9,10]. As *e2f1* is a key transcription factor of G1/S transition, we were interested in studying whether it is under control of Wnt/β-catenin/Tcf signaling pathway. Here, we identified two putative Tcf-binding elements within the *e2f1* gene promoter and checked a proximal element, which is partially overlapped with E2F-binding B-element [11], for Tcf-dependent regulation. Our data demonstrate that this element effectively competes with a canonical element from *cyclin D1* promoter for binding in a gel retardation assay. Moreover, being inserted into minimal TA-luc reporter it provides positive transcriptional regulation in response to co-transfection of Tcf/β-catenin expression vectors and treatment with HDAC inhibitors.

Materials and methods

Cells and reagents. Mouse embryo fibroblasts were transformed with complementing oncogenes E1A and cHa-Ras (mERas cells) [10]. Sodium butyrate was purchased from Sigma, Trisol reagent

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and Lipofectamine-2000 from Invitrogen, dual-luciferase kit was from Promega, oligonucleotides were synthesized in Syntol (Russia). The E2F1 human promoter (−242 bp)-luciferase construct and its mutants into either of two putative E2F recognition sites were kindly provided by Dr. Ikeda. Other plasmids were kindly donated by Dr. K.W. Kinzler and Dr. Bert Vogelstein (β-catenin and Tcf-4 expression vectors), Dr. M. Bordonaro (pTOP-Flash and pFOP-Flash reporter plasmids).

Cell cycle analysis. Cells were washed and permeabilized for 30 min with 0.01% saponin. Then the cells were washed with PBS and incubated for 15 min at 37 °C with 40 μg/ml propidium iodide, 0.1 mg/ml RNase A prior to analysis using an ATC300 cytometer (Brucker).

RT-PCR. The total cellular RNA was isolated by Trisol. The RT step was performed with 3 μg of RNA and 1 μg of random hexaprimers (Roche Applied Science). The amount of total RNA for RT-reactions was normalized by densitometry of 18S ribosomal RNA band after electrophoresis in denaturing agarose gel. The PCR step was performed in the presence of 100 ng of primers to the cDNA of mouse genes: *e2f1* (5′-TGGTAGCAGTGGGCCATTCC-3′/5′-GCCGTTTC TGCACCTTCAGC-3′); *β-catenin* (5′-CCGGCCATTGTAGAAGCTGG-3′/ 5′-CCATCTCGGCTTCCTGATGC-3′) and *gapdh* as an internal control (5′-TGTGATGGGTGTGAACCACG/5′-CCAGTGAGCTTCCCGTTCAG-3′).

Plasmid construction and transfection. Proximal Tcf-binding element of *e2f1* promoter (shown in bold and italics) was flanked by linker sites for KpnI and BglII restriction nucleases to insert it into polylinker of TA-luc reporter (Clontech Lab): 5′-***CGTAAAAGTGGCCGG GACTTTGCA***A-3′; 3′-CATG***GCATTTTCACCGGCCCTGAAACGT***TCTAG-5′.

The ligated construct (TA-Tcf-luc) was cloned in *Escherichia coli* HB101. Purified plasmid DNA (1 μg) was used to transiently transfect mERas cells with Lipofectamine-2000 according to a protocol of manufacturer. Cells were co-transfected with vectors expressing β-catenin or Tcf proteins (1 μg). DNA content was equalized by DNA of pUC19 plasmid. DNA of *Renilla* (0.2 μg) was used as an internal control of transfection. Transfection of mERas cells with TOP-flash and FOP-flash reporters has been performed similarly. TOP-flash construct bears luciferase reporter under canonic Tcf-regulated promoter from *cyclin D1* gene. FOP-flash is the same construct but containing mutation in Tcf-binding site. TOP-flash to FOP-flash activity ratio was used to characterize changes in Tcf activity. In 24–48 h after transfection cells were processed to measure luciferase activity according to recommendation of manufacturer. Analysis of luciferase activity was done on luminometer TD-20/20 (Turner Designs). Each experiment was repeated at least three times. Diagrams represent means ± SEM.

DNA-binding analysis. Nuclear extracts were used in a gel retardation assay as described [12]. Reaction mixture for DNA binding included 5 mM HEPES, pH 7.9, 1 mM dithiothreitol, 2.5 mM EDTA, 2.5 mM $MgCl_2$, 15% glycerol and 5 μg of nuclear extracts. The mixtures were incubated at room temperature for 20 min, and then ^{32}P-labeled oligonucleotide was added (30,000 cpm/ng) and incubation was continued for an additional 20 min. The Tcf-binding sites from the *e2f1* and *cyclin D1* promoters used as double-strand probes for retardation were: 5′-TGC AAA GTC CCG GCC ACT TTT ACG-3′ and 5′-GTT AAG CAG AGA TCA AAG CCG GGC-3′, respectively.

Specific and non-specific oligonucleotides were added in competition experiments at a 100-fold molar excess over the labeled probe. To verify the complex specificity, antibodies to β-catenin (E-5) and Tcf-4 (H-125)X (Santa Cruz Biotech) proteins were added 30 min prior of the labeled oligonucleotide. DNA–protein complexes were separated by electrophoresis on 4% polyacrylamide gel (30:1) using Tris–borate buffer, pH 8.3, as an electrode buffer. Oligonucleotides were labeled by polynucleotide kinase in the presence of [γ-^{32}P]ATP.

Results

Promoter of e2f1 gene comprises nucleotide sequences capable of Tcf binding

HDAC inhibitors sodium butyrate (NaB) and Trichostatin A (TSA) induce G1/S arrest of E1A + Ras transformed MEF cells (mERas cells) as evidenced by cell cycle distribution and suppression of cell growth ([10] and data not shown). Among genes directly involved in G1–S progression like *c-myc* and *cyclin D1*, the NaB-arrested cells demonstrate down-regulation of *e2f1* gene, which plays a key role in G1/S transition (Fig. 1A, left panel). But the mechanism of *e2f1* gene repression remains unclear. The *e2f1* gene promoter contains a number of regulatory elements, in particular several Sp-1 sites, ATF and two E2F-binding sites: the distal element A (position −31 to −20) and closely located the proximal element B (position −14 to −3) [11,13]. Analysis of the sequence of *e2f1* gene promoter reveals that, besides two E2F-responsive elements, it also contains two tandems of putative not yet identified Tcf-binding elements thus potentially allowing Tcf-dependent regulation of *e2f1* transcription (Fig. 1B). Moreover, the proximal Tcf elements, located at −15/+8 bp of transcription site, are partially overlapped with E2F-binding element (−23/−11) and may therefore interfere with E2F auto-regulation loop. The identified putative elements were similar to canonic Tcf-binding sequences but had a few mismatches. Therefore, it was important to test whether they are actually able to bind Tcf complexes.

We used a gel retardation assay to address this question. These results demonstrated that the labeled proximal Tcf element competed well with a canonical Tcf element of *cyclin D1* promoter for Tcf binding (Fig. 1C). The same result was obtained in reciprocal experiment where labeled canonical Tcf-binding site competed with *e2f1*-derived Tcf element (data not shown). Specificity of complex formation was checked by addition of antibodies to β-catenin and Tcf proteins, which have been shown to decrease the intensity of complexes (Fig. 1D, right panel).

Besides, we found that NaB-treated mERas cells reveal increased transcription of *β-catenin* gene (Fig. 1A, right panel) accompanied by nuclear accumulation of β-catenin protein [10]. β-Catenin protein exerts its co-activator function in complexes with Tcf and other factors thereby regulating transcription of various genes including *c-myc* and *cyclin D1* [2,3]. In spite of DNA binding activity of proximal Tcf element is found to slightly change in NaB-treated cells (Fig. 1D) one can see an increase of Tcf-transactivation with TOP-flash reporter driven by Tcf-responsive element (Fig. 1E).

Collectively, the obtained data demonstrate that *e2f1* gene promoter contains a nucleotide sequence that is capable of binding with Tcf/β-catenin complexes and therefore making *e2f1* a potential target of Wnt/β-catenin/Tcf pathway. These results allow suggesting Tcf-dependent down-regulation of *e2f1* gene transcription in NaB-treated cells.

Proximal Tcf-binding element of e2f1 promoter provides transcriptional regulation by Tcf/β-catenin and gets activated after NaB treatment

Araki et al. [11] identified two E2F-binding sites in the *e2f1* promoter which play distinct roles in the regulation of *e2f1* transcription by interacting with different sets of E2F members: E2F4/p130 repressor complex specifically binds to the distal E2F-binding site, whereas E2F1 and E2F3 activators preferentially bind to the proximal E2F-binding site. Control experiments showed that transfection of E2F-expression vector increased activity of e2f1-luc reporter (data not shown). We have also checked activity of the luciferase reporter driven by *e2f1* promoter after co-transfection

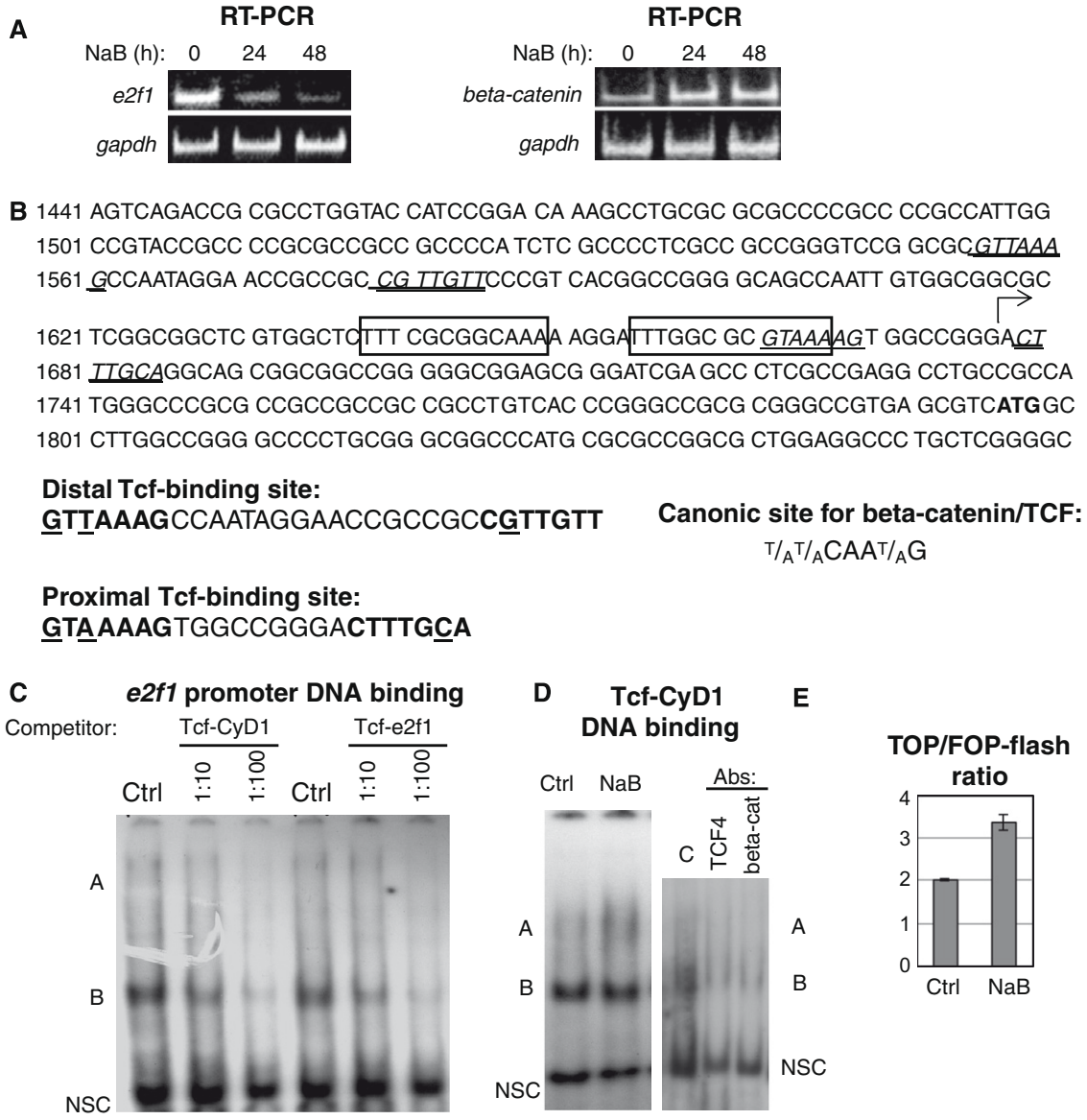

Fig. 1. NaB down-regulates *e2f1* and up-regulates *β-catenin* transcription in mERas cells. (A) RT-PCR analysis of *e2f1* (left panel) and *β-catenin* (right panel) transcripts in mERas cells treated with 4 mM NaB for 24 and 48 h. (B) Identification of putative Tcf-responsive elements in the *e2f1* promoter. Sequence of *e2f1* gene promoter is taken from NCBI Nucleotide database (http://www.ncbi.nlm.nih.gov, reference sequence NC_000068.6). In addition to two E2F-responsive elements, the *e2f1* promoter contains two putative Tcf-binding elements (mismatched nucleotides underlined), which represent tandem repeats containing a spacer within. β-Catenin/TCF-binding elements are shown by underlined italics, E2F-binding elements are boxed, ATG start-codon is bold. Upstream half-element of proximal Tcf is partially overlapped with the proximal E2F-binding element. (C) DNA binding activity of the proximal Tcf element of *e2f1* promoter (Tcf-e2f1): competition with unlabeled canonical Tcf element from *cyclin D1* promoter (Tcf-CyD1) and from e2f1 promotor (Tcf-e2f1). A and B – Tcf-specific complexes, NSC – non-specific complex. (D) DNA binding activity of Tcf complexes in nuclear extracts from NaB-treated mERas cells (24 h) (left panel). A and B – Tcf-specific complexes, NSC – non-specific complex. Right panel describes specificity of the complex formation after addition of antibodies to β-catenin and Tcf proteins. (E) NaB activates Tcf-responsive TOP-flash reporter. mERas cells were transfected with TOP-flash (canonical Tcf element) and FOP-flash (mutated Tcf element) plasmids and the ratio of TOP/FOP is given.

with vectors expressing Tcf and β-catenin. Data presented in Fig. 2A show that the construct is negatively regulated by Tcf/β-catenin complexes as well as by NaB.

The fact of physical interaction of *e2f1* promoter elements with Tcf proteins on its own is not sufficient for concluding that this promoter is actually regulated by Tcf/β-catenin complexes and moreover that its activity is modulated by NaB. To test this suggestion we inserted proximal Tcf-binding element into minimal reporter vector TA-luc (Clontech Lab, see Materials and methods). Co-transfection of the TA-Tcf-luc reporter with Tcf and β-catenin encoding plasmids has shown that described Tcf-binding element provides positive regulation by Tcf/β-catenin (Fig. 2B). Furthermore, we have found that identified Tcf element provides an activation of reporter gene transcription in response to NaB treatment (Fig. 2C), similarly to canonical Tcf-site in TOP-flash reporter (Fig. 1E).

All these results allow concluding that *e2f1* gene promoter contains a Tcf/β-catenin-responsive element that makes NaB able to modulate *e2f1* gene transcription through Wnt/β-catenin/Tcf signaling pathway.

Discussion

E2F transcription factor is a key regulator of cell cycle through the control of G1/S transition. This regulation is provided by E2F-binding with inhibitory proteins of retinoblastoma (Rb) family,

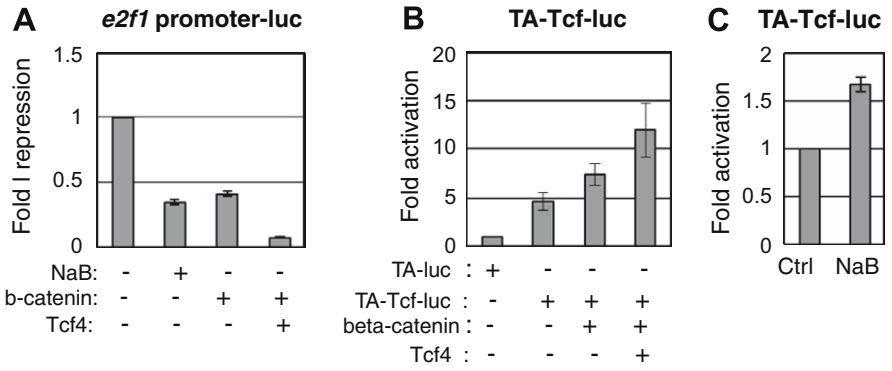

Fig. 2. Activity of luciferase reporters driven by *e2f1* gene promoter (A) is negatively regulated by Tcf/β-catenin. Cells were co-transfected with vectors expressing β-catenin/Tcf-4 proteins. (B) Proximal *e2f1*-Tcf element provides positive regulation in the content of TA-Tcf-luc reporter in mERas cells transfected with Tcf-4 and β-catenin expressing vectors. (C) NaB activates TA-Tcf-luc reporter containing proximal *e2f1*-Tcf element. Cells were treated by NaB or left untreated for 24 h.

which undergo phosphorylation at the end of G1 phase leading to E2F release and subsequent activation of target proliferation-specific genes [14].

But recently it was found that the role of E2F is much wider than just cell division control. This transcription factor was also shown to participate in the regulation of such important biological processes as differentiation and development [15], apoptosis [16] and RNA processing [17]. This broad range of functions requires more complex and accurate regulation of E2F activity. Indeed, E2F1 activity is regulated not only due to its interaction with other proteins but also through modulation of *e2f1* gene transcription. Moreover, we showed that NaB and TSA down-regulate *e2f1* transcription but up-regulate transcription of *β-catenin* gene (Fig. 1 and [10,18]). In line with this, we identified Tcf-responsive elements within the *e2f1* promoter, thus, demonstrating that *e2f1* gene may belong to a number of genes regulated through Wnt/β-catenin pathway. This pathway plays an important role in development, cell proliferation and differentiation. Thus, our finding helps to shed some light on the link between E2F and Wnt signaling.

The *e2f1* gene expression in E1A + Ras-transformed cells can be suppressed by NaB and TSA due to activation of Wnt/β-catenin/Tcf pathway. In support to our data, Sun et al. [19] obtained results that inhibitors of GSK3β LiCl, TDZD-8 and siRNA, which activate the Wnt signaling pathway, significantly decreased the expression of multiple DNA replication-related genes, including *cdc6*, *cyclin A*, *cyclin E*, and *cdc25C* regulated by E2F factor during the cell cycle. Furthermore, LiCl treatment suppressed E2F transactivation by interrupting the interaction of E2F1 factor with its target gene promoter [19]. The identified here Tcf-responsive elements being inserted into TA-Luc reporter provided positive regulation of the reporter by NaB and exogenous Tcf/β-catenin. Noteworthy that natural *e2f1* promoter is under negative regulation by these factors. This might be explained by disruption of interaction at the promoter due to interfering binding the Tcf/β-catenin complexes with overlapping E2F and Tcf elements. Recently, it has been shown that LEF-1 protein of the Tcf/LEF family can interact with E2F1 transcription factor, thus affecting its binding with *e2f1* gene promoter and altering its transcriptional auto-regulation [20]. These data support the idea that transactivational potential of Tcf/β-catenin complexes depends on the nucleotide context and on Tcf binding with other proteins.

Importantly, it was also recently found that E2F1 protein itself could repress β-catenin/Tcf activity [21,22], thus providing a positive feedback loop, which in our case might trigger G1/S cell cycle arrest of mERas cells. But at the same time, reverse activation of this feedback loop, to the contrary, is potentially able to facilitate tumor development [21]. Thus, for the first time our study demonstrates Wnt-dependent *e2f1* regulation which implies a link between E2F and Wnt pathways.

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