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β-Catenin/TCF/LEF regulate expression of the short form human Cripto-1

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Abstract

The human gene *Teratocarcinoma-derived growth factor 1 (TDGF1)/Cripto-1/CR-1* which is expressed in a wide variety of human carcinomas is a member of the *EGF-cripto FRL1 cryptic (EGF-CFC)* gene family. A majority of human colorectal tumors and hepatomas are known to possess a constitutively active canonical *Wnt*/β-catenin/TCF signaling pathway, also express CR-1. Expression of a short form of CR-1 mRNA in colon carcinoma and hepatoma cell lines suggests that there may be differential regulation of CR-1 expression by the canonical *Wnt* signaling pathway in colon cancer as well as hepatoma cell lines. The present study demonstrates a direct transcriptional regulation of the short form CR-1 expression by the canonical *Wnt* signaling pathway through an intronic–exonic enhancer element, containing three tandem TCF/LEF binding sites within the *CR-1* gene.

Keywords: *Wnt*; Cripto-1; β-Catenin

Teratocarcinoma-derived growth factor 1 (TDGF1/Cripto-1/CR-1) is a member of the *epidermal growth factor-cripto FRL1 cryptic (EGF-CFC)* gene family and essential for Nodal/Gdf1/3 signaling as a co-receptor [1,2]. In addition to functioning as a Nodal co-receptor, CR-1 can activate mitogen-activated protein kinase (MAPK), Akt and c-src intracellular signaling pathways independently of Nodal and ALK4 [3]. Several types of human cancers including colorectal tumors and hepatomas overexpress CR-1 [4], and CR-1 can also function as an oncogene *in vitro* and *in vivo* [5–7]. The constitutively active *Wnt* signaling pathway that is mediated by β-catenin/TCF/LEF complex has important role in the pathogenesis of colorectal tumors and hepatomas [8]. This evidence suggests that there might be a functional relationship between CR-1 and the *Wnt* signaling pathway in these cells where CR-1 might be a *Wnt* target gene [9]. However, a detailed delineation as to the transcriptional mechanism by which *Wnt* signaling pathway might regulate CR-1 expression is still unclear.

Colon carcinoma, hepatoma cell lines and primary human colon cancers express a truncated form of CR-1 mRNA [10]. The truncated CR-1 mRNA lacks exon 1 and 2, but the putative open reading frame still maintains the EGF-like module, the cysteine-rich domain and the COOH-terminal linkage sequence. Although the biological effects of this truncated form of CR-1 have not yet been determined, the fact that the short form of CR-1 is a major transcript in such tumors suggests the importance of short form CR-1 (SF-CR1) in cancer progression.

In this study, we focused on the transcriptional regulation of *CR-1* by the canonical *Wnt* signaling pathway in colon carcinoma and hepatoma cell lines, and confirmed direct regulation through an enhancer element located within the *CR-1* gene itself. These findings provide novel detailed information on the complex regulation of this gene, and suggest a possible cancer-specific therapeutic target.

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Materials and methods

Cell culture. The culture condition of each cell line was as follows: NT2/D1 human embryonic carcinoma cell line: McCoy's 5A media with 15% fetal bovine serum (FBS), Ls174T human colon cancer cell line: Minimum Essential Media (MEM) with 10% FBS, SW620 human colon cancer cell line: Leibovitz's L-15 media with 10% FBS, HepG2 human hepatoma cell line: MEM with 10% FBS and 1 mM sodium pyruvate, COS-7: Dulbecco's modified Eagle's media with 10% FBS.

5′-Rapid amplification of cDNA ends (RACE). 5′RACE was performed using SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA). The amplified 5′ cDNA end of CR-1 mRNA in Ls174T was subcloned and confirmed by direct sequencing.

Real-time PCR. Real-time PCR was performed by using a LightCycler (Roche Applied Science, Indianapolis, IN) and QuantiTect SYBR Green PCR Kit (QIAGEN, Valencia, CA). The primer set for GAPDH was as follows; GAPDH F: 5′ CAGGAGCGAGATCCCT 3′, GAPDH R: 5′ GGTGCTAAGCAGTTGGT 3′. The total CR-1 primer and full-length CR-1 specific primer sets were described previously [11,12]. The normalization method has been described previously [11].

Construction of vectors. The Lef-1 cDNA was PCR amplified and cloned into the pUSE expression vector (UPSTATE, Chicago, IL). The TCF4 expression vector was purchased from UPSTATE. The constitutively active Ser33Tyr β-catenin expression vector was made by site-directed mutagenesis from WT-β-catenin in pFLAG-CMV-2 vector [13]. The following DNA fragments were cloned into pGL4.14 firefly luciferase vector (Promega, Madison, WI); pGL4 10K (−8400 to +1632; ATG of full length CR-1 is defined as 0); pGL4 9K (−7794 to +1632); pGL4 1.3K (+313 to +1632); pGL4 INT (+778 to +1364). pEF1α-RL was constructed by ligating Renilla luciferase into pEF1 V5His empty vector (Invitrogen, Carlsbad, CA). QuikChange Multi Site-Directed Mutagenesis Kit (STRATAGENE, La Jolla, CA) was used to introduce mutated TCF/LEF binding element (TBE) sequences.

Dual-luciferase assay. For the luciferase assay, 100 ng/well of indicated reporter vectors, 10 ng/well PEF1α-RL vector and the indicated amount of expression vectors or empty vector (pcDNA3.1V5His vector, Invitrogen) were transfected by lipofection. Luciferase assay was carried out by using Dual-Luciferase Reporter Assay Kit (Promega).

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was done by using ChIP assay Kit (UPSTATE). A DNA/protein complex was sheared by sonication. One percent of sheared DNA/protein complex was kept as an input DNA sample. Anti-TCF4 mouse monoclonal antibody (clone 6H5-3, UPSTATE), or normal mouse IgG (UPSTATE) was used for immunoprecipitation. Retrieved DNA was analyzed by PCR analysis by using the following primer set;

INT Fw: 5′ GAAAGATGGTGCTCTACAATTTCTT 3′,
INT Rev: 5′ AATGGCCATGATCCAAATCACACTA 3′.

RNA interference. For the transient β-catenin knockdown, siGENOME SMARTpool CTNNB1 (β-catenin) and siCONTROL Non-Targeting siRNA Pool (M-003482-00 and D-001206-13-20, Dharmacon, Lafayette, CO) were used.

Western blot analysis. Western blot analysis was done as described previously [3]. Primary antibodies were as follows; β-catenin (06-734, UPSTATE), β-actin (clone AC-15, Sigma–Aldrich, St. Louis, MO, USA), E-cadherin (SC8426, Santa Cruz Biotechnology, Santa Cruz, CA) and N-cadherin (SC7939, Santa Cruz Biotechnology). Specific bands were quantified using NIH Image Software (NIH, Bethesda, MD).

Results and discussion

The short form CR-1 mRNA is dominantly expressed in Wnt-active cell lines

Previous studies have shown that the short form CR-1 (SF-CR1) mRNA is expressed in Ls174T, SW620 and HepG2 cells [10]. At first, we confirmed the transcription start site of SF-CR1 by 5′RACE and identified an alternative transcription start site within exon 3 that corresponds to 385 bp of full-length CR-1 mRNA (Fig. 1A). Since these cell lines are reported to have an activated canonical *Wnt*-pathway [9] [14,15], we analyzed 10 kb upstream of the human genomic sequence of *CR-1* gene from exon 3 for TCF/LEF binding elements (TBE). The database analysis was done by Genomatix software application based on the DNA sequence of GenBank Accession No. AC104304. Multiple TBEs were identified (Fig. 1A), including the intronic–exonic three tandem repeat of TBE, which is located in the proximity of the transcriptional start site of SF-CR1. To quantify the full-length CR-1

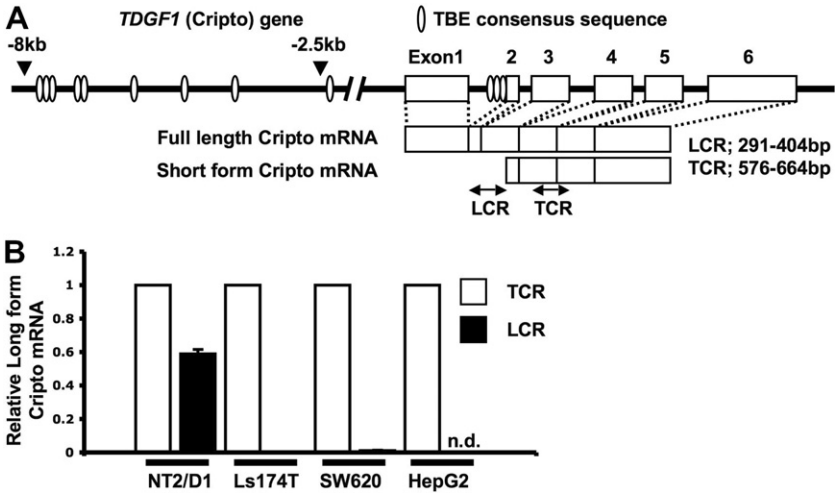

Fig. 1. The *TDGF1 (CR-1)* gene and mRNA structure/*CR-1* isoform expression in human cancer cells. (A) *TDGF1 (CR-1)* gene and mRNA structure. LCR; full-length CR-1 specific primer set, TCR; total CR-1 primer set. (B) Quantification of full-length CR-1 within total CR-1 by real-time PCR ($n = 3$). The relative full-length CR-1 expression level in each cell line was normalized by total CR-1 expression level. n.d., not detectable.

mRNA within the total CR-1 transcript, we used a full-length CR-1 specific primer set (LCR) and total CR-1 primer set (TCR) that reacts to full-length and SF-CR1 (Fig. 1A). As shown in Fig. 1B, 60% of the total CR-1 transcripts were represented by full-length CR-1 in NT2/D1 cells. In contrast, in Ls174T, SW620, and HepG2 cells full-length CR-1 transcript expression was significantly lower (less than 2%) than NT2/D1 cells, suggesting that the majority of CR-1 transcripts represent the SF-CR1.

A recent report has demonstrated that the *CR-1* pseudogene *CRIPTO-3*-derived transcript that is located on chromosome X is also detectable in NT2/D1 cells [12]. This is due to the high similarity of the proximal 5′ flanking region of the *CR-1* gene exon 1 and *CRIPTO-3* gene [12]. We also assessed *CRIPTO-3* expression in HepG2 and SW620 cells by realtime-PCR using a *CRIPTO-3*-specific primer set. Compared to the TCR primer set, *CRIPTO-3* expression was much less abundant (less than 3% of total CR-1 transcript) in both cell lines (data not shown).

The intronic–exonic TBEs contribute to transcriptional regulation of CR-1 by a canonical Wnt signaling pathway

We assessed the transcriptional regulation of the *CR-1* promoter by luciferase assay. As shown in Fig. 2A, co-transfection of constitutively active β-catenin with TCF4 or Lef-1 significantly activated pGL4 10K reporter in COS-7 cells as compared to the luciferase activity in COS-7 cells transfected with only pGL4 10K. Following deletion of upstream TBEs, pGL4 9K, pGL4 1.3K and pGL4 INT reporters were still capable of being activated by the canonical *Wnt*-pathway. To confirm that these effects were mediated by the three intronic–exonic TBEs, we mutated the intronic TBEs in pGL4 INT reporter as shown in Fig. 2B (mutated sequences are underlined). The TBE1 mutation had no effect, but TBE2 or TBE3 mutation significantly suppressed reporter activation (Fig. 2B). Since mutation of all three TBEs resulted in the reporter activity returning to the basal level, we concluded that intronic–exonic TBEs are cooperatively regulated by the canonical *Wnt*-pathway.

The intronic–exonic TBEs are responsible for the high basal activity of intronic reporter in Wnt/β-catenin/TCF activated cell lines

The basal activity of the pGL4 INT reporter was also confirmed in HepG2 and SW620 cells. The pGL4 INT reporter showed high basal activity in both cell lines (Fig. 3A). Mutation of each TBE also attenuated basal reporter activity in both cell lines, and mutations of all three TBEs resulted in lower reporter activity. To confirm endogenous direct binding of β-catenin/TCF4 complex to the intronic–exonic TBEs, we performed ChIP assays by using a monoclonal TCF4 antibody that has been shown to co-immunoprecipitate β-catenin in a *Wnt*-active cell line [16]. The intronic–exonic enhancer element containing DNA fragment was immunoprecipitated in both HepG2 and SW620 cells (Fig. 3B), which showed direct binding of the TCF4 to this enhancer element. These results also support a dominant function of the enhancer element within the *CR-1* gene in HepG2 and SW620 cells, which is not completely conserved in the *CRIPTO-3* gene.

siRNA-against β-catenin represses CR-1 expression

We confirmed the contribution of canonical *Wnt* signaling in the transcriptional regulation of *CR-1* in HepG2 and

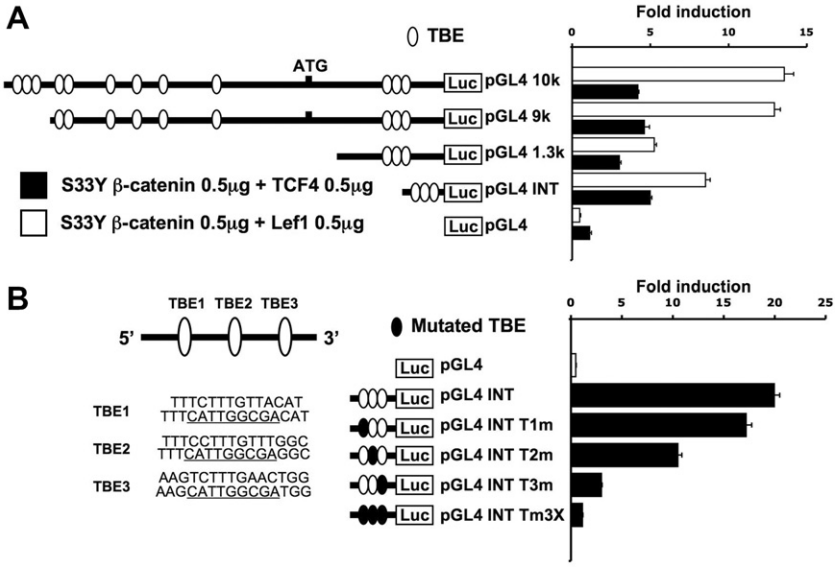

Fig. 2. The CR-1 gene promoter regulation by canonical *Wnt* signaling pathway. (A) Cotransfection assays and deletion assays (COS-7, $n = 3$). Each reporter's activity was normalized by corresponding reporter activity co-transfected with 1 μg of empty vector. (B) Mutation analysis (COS-7, $n = 3$). Each reporter's activity was normalized by corresponding reporter activity co-transfected with 1 μg of empty vector.

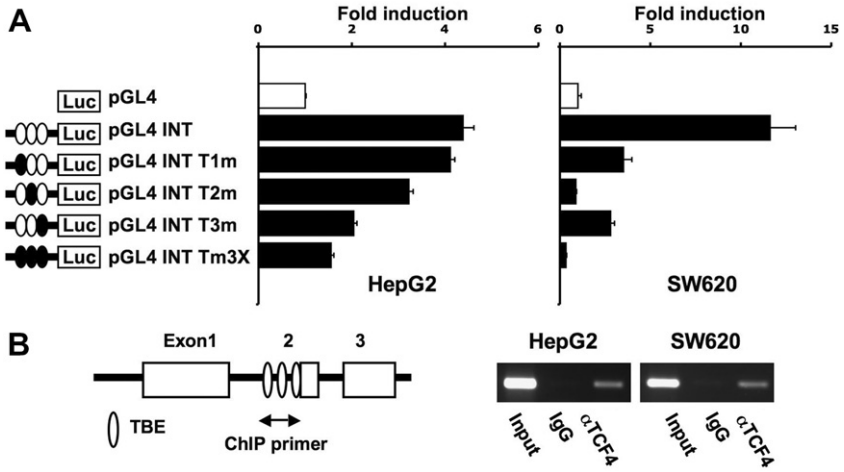

Fig. 3. The intronic–exonic enhancer is directly regulated by canonical *Wnt* signaling pathway in SF-CR1 expressing cells. (A) Basal activity of wild-type and TBE-mutated reporter constructs in HepG2 and SW620 cells ($n = 3$). Each reporter's activity was normalized by the pGL4 activity. (B) ChIP assay of the intronic–exonic enhancer element. The anti-TCF4 antibody immunoprecipitated the intronic–exonic enhancer element containing DNA. Normal mouse IgG was used for control immunoprecipitation.

SW620 cells by an si-RNA-based method. As shown in Fig. 4A, both HepG2 and SW620 cells treated with siRNA against β-catenin showed a strong decrease in total β-catenin expression by Western blot analysis, as assessed by densitometric analysis. There was a 40–50% decrease in β-catenin protein expression in both cell lines (Fig. 4A). By real-time PCR analysis (using the TCR primer set), CR-1 expression was repressed up to 50% (Fig. 4B) in β-catenin siRNA-treated cells, which indicates significant contribution of endogenous *Wnt* signaling to CR-1 expression in these cell lines.

At the same time, β-catenin siRNA treatment induced E-cadherin expression and repressed N-cadherin expression in HepG2 cells (Fig. 4C), suggesting a biochemical change to a more epithelial state. This result also suggests the possible involvement of SF-CR1 to the *Wnt*-induced

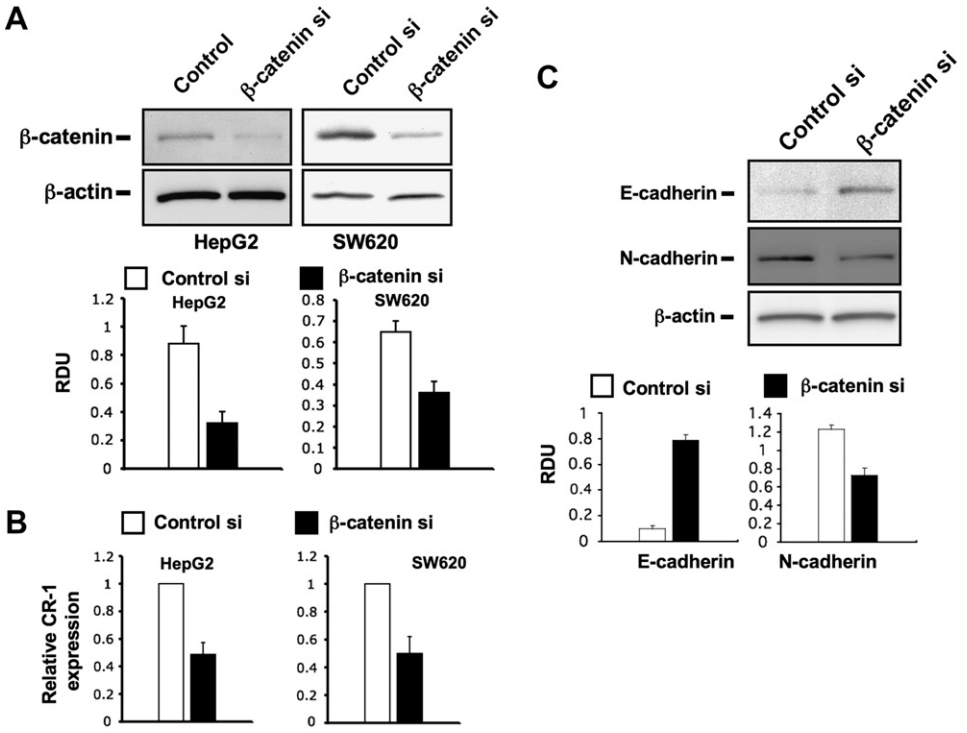

Fig. 4. β-Catenin knockdown repressed CR-1 expression. (A) Western blot for β-catenin in siRNA-treated HepG2 and SW620 cells ($n = 5$). RDU; relative densitometry unit. (B) Real-time PCR analysis for CR-1 mRNA ($n = 5$). The relative CR-1 expression level was normalized by GAPDH expression level. (C) Western blots for E-cadherin and N-cadherin in siRNA-treated HepG2 cells ($n = 3$).

EMT in cancer cells, since a previous study has shown that CR-1 can induce EMT in mouse mammary epithelial cells [7]. The biological effect of the SF-CR1 has not yet been elucidated, but peptides corresponding to the EGF-like module of CR-1 have been shown to stimulate cell proliferation in NT2/D1 cells [17]. Since deregulation of the canonical *Wnt* signaling pathway is reported in a wide variety of human cancers, this *Wnt*-dependent transcriptional regulation of *CR-1* observed in colon and hepatoma cells could represent a potential cancer-specific therapeutic target.

References

[1] E. Reissmann, H. Jornvall, A. Blokzijl, O. Andersson, C. Chang, G. Minchiotti, M.G. Persico, C.F. Ibanez, A.H. Brivanlou, The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development, Genes. Dev. 15 (2001) 2010–2022.

[2] C. Bianco, H.B. Adkins, C. Wechselberger, M. Seno, N. Normanno, A. De Luca, Y. Sun, N. Khan, N. Kenney, A. Ebert, K.P. Williams, M. Sanicola, D.S. Salomon, Cripto-1 activates nodal- and ALK4-dependent and -independent signaling pathways in mammary epithelial cells, Mol. Cell. Biol. 22 (2002) 2586–2597.

[3] C. Bianco, L. Strizzi, A. Rehman, N. Normanno, C. Wechselberger, Y. Sun, N. Khan, M. Hirota, H. Adkins, K. Williams, R.U. Margolis, M. Sanicola, D.S. Salomon, A Nodal- and ALK4-independent signaling pathway activated by Cripto-1 through Glypican-1 and c-Src, Cancer Res. 63 (2003) 1192–1197.

[4] C. Bianco, L. Strizzi, N. Normanno, N. Khan, D.S. Salomon, Cripto-1: an oncofetal gene with many faces, Curr. Top. Dev. Biol. 67 (2005) 85–133.

[5] C. Wechselberger, L. Strizzi, N. Kenney, M. Hirota, Y. Sun, A. Ebert, O. Orozco, C. Bianco, N.I. Khan, B. Wallace-Jones, N. Normanno, H. Adkins, M. Sanicola, D.S. Salomon, Human Cripto-1 overexpression in the mouse mammary gland results in the development of hyperplasia and adenocarcinoma, Oncogene 24 (2005) 4094–4105.

[6] Y. Sun, L. Strizzi, A. Raafat, M. Hirota, C. Bianco, L. Feigenbaum, N. Kenney, C. Wechselberger, R. Callahan, D.S. Salomon, Overexpression of human Cripto-1 in transgenic mice delays mammary gland development and differentiation and induces mammary tumorigenesis, Am. J. Pathol. 167 (2005) 585–597.

[7] L. Strizzi, C. Bianco, N. Normanno, M. Seno, C. Wechselberger, B. Wallace-Jones, N.I. Khan, M. Hirota, Y. Sun, M. Sanicola, D.S. Salomon, Epithelial mesenchymal transition is a characteristic of hyperplasias and tumors in mammary gland from MMTV-Cripto-1 transgenic mice, J. Cell. Physiol. 201 (2004) 266–276.

[8] R.H. Giles, J.H. van Es, H. Clevers, Caught up in a Wnt storm: Wnt signaling in cancer, Biochim. Biophys. Acta. 1653 (2003) 1–24.

[9] M. Morkel, J. Huelsken, M. Wakamiya, J. Ding, M. van de Wetering, H. Clevers, M.M. Taketo, R.R. Behringer, M.M. Shen, W. Birchmeier, Beta-catenin regulates Cripto- and Wnt3-dependent gene expression programs in mouse axis and mesoderm formation, Development 130 (2003) 6283–6294.

[10] G. Baldassarre, M. Tucci, G. Lembo, F.M. Pacifico, R. Dono, C.T. Lago, A. Barra, C. Bianco, G. Viglietto, D. Salomon, M.G. Persico, A truncated form of teratocarcinoma-derived growth factor-1 (cripto-1) mRNA expressed in human colon carcinoma cell lines and tumors, Tumour Biol. 22 (2001) 286–293.

[11] C. Bianco, L. Strizzi, M. Mancino, A. Rehman, S. Hamada, K. Watanabe, A. De Luca, B. Jones, G. Balogh, J. Russo, D. Mailo, R. Palaia, G. D'Aiuto, G. Botti, F. Perrone, D.S. Salomon, N. Normanno, Identification of cripto-1 as a novel serologic marker for breast and colon cancer, Clin. Cancer Res. 12 (2006) 5158–5164.

[12] M. Hentschke, I. Kurth, U. Borgmeyer, C.A. Hubner, Germ cell nuclear factor is a repressor of CRIPTO-1 and CRIPTO-3, J. Biol. Chem. 281 (2006) 33497–33504.

[13] L.N. Song, R. Herrell, S. Byers, S. Shah, E.M. Wilson, E.P. Gelmann, Beta-catenin binds to the activation function 2 region of the androgen receptor and modulates the effects of the N-terminal domain and TIF2 on ligand-dependent transcription, Mol. Cell. Biol. 23 (2003) 1674–1687.

[14] X. Huang, B. Guo, Adenomatous polyposis coli determines sensitivity to histone deacetylase inhibitor-induced apoptosis in colon cancer cells, Cancer Res. 66 (2006) 9245–9251.

[15] A. de La Coste, B. Romagnolo, P. Billuart, C.A. Renard, M.A. Buendia, O. Soubrane, M. Fabre, J. Chelly, C. Beldjord, A. Kahn, C. Perret, Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas, Proc. Natl. Acad. Sci. USA 95 (1998) 8847–8851.

[16] T. Ishitani, J. Ninomiya-Tsuji, S. Nagai, M. Nishita, M. Meneghini, N. Barker, M. Waterman, B. Bowerman, H. Clevers, H. Shibuya, K. Matsumoto, The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF, Nature 399 (1999) 798–802.

[17] G. Baldassarre, A. Romano, F. Armenante, M. Rambaldi, I. Paoletti, C. Sandomenico, S. Pepe, S. Staibano, G. Salvatore, G. De Rosa, M.G. Persico, G. Viglietto, Expression of teratocarcinoma-derived growth factor-1 (TDGF-1) in testis germ cell tumors and its effects on growth and differentiation of embryonal carcinoma cell line NTER-A2/D1, Oncogene 15 (1997) 927–936.