Identification of IGFBP-6 as a significantly downregulated gene by β-catenin in desmoid tumors

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Desmoid tumors (aggressive fibromatosis) are locally invasive soft tissue tumors in which β-catenin-mediated TCF-3-dependent transcription is activated. To provide more insight into the pathophysiology of these tumors, expression profiles were generated using oligonucleotide arrays (Affymetrix). In total, 69 differentially expressed genes were identified in desmoids compared to normal fibroblasts (fascia) from the same patients. The differential expression of a selection of genes was confirmed using RT–PCR and Northern blotting. We further evaluated the insulin-like growth factor-binding protein 6 (IGFBP-6), a gene that was consistently downregulated in all desmoids tested. Promotor studies and electromobility shift assays revealed two functional β-catenin/TCF-responsive elements in the human IGFBP-6 promoter. These findings suggest that IGFBP-6 is directly downregulated by the β-catenin/TCF complex in desmoid tumors, and imply a role for the IGF axis in the proliferation of desmoid tumors.
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Introduction

Desmoid tumors (also called aggressive fibromatosis) are locally invasive, benign soft-tissue tumors, composed of fibroblast-like cells that arise from fascia or musculoaponeurotic structures. These tumors can occur as sporadic lesions or as a part of familial adenomatous polyposis, which is caused by germline mutations in the adenomatous polyposis coli (APC) gene (Eccles *et al.*, 1996). Sporadic desmoids harbor somatic mutations in either the APC gene or in the β-catenin gene, resulting in β-catenin protein stabilization and nuclear accumula-

tion (Alman *et al.*, 1997; Li *et al.*, 1998; Tejpar *et al.*, 1999). Using transgenic mice expressing conditional stabilized β-catenin, it was recently demonstrated that β-catenin stabilization in fibroblasts is sufficient to cause aggressive fibromatosis (Cheon *et al.*, 2002).

β-catenin is a key component of the Wnt signaling pathway. Upon Wnt signaling or through oncogenic mutations, the β-catenin protein is stabilized, accumulates and translocates to the nucleus, where it interacts with members of the TCF/Lef family of transcription factors to modulate the transcription of target genes (Morin *et al.*, 1997). Four different members of the TCF family have been detected in humans: TCF-1, Lef-1, TCF-3, and TCF-4 (Cadigan and Nusse, 1997). In colorectal cancer, nuclear β-catenin forms a complex with TCF-4 and activates target genes, such as c-myc, cyclin D1, MMP7, fra1, c-jun, and PPAR delta (He *et al.*, 1998).

Previously, we demonstrated constitutive TCF activation in primary desmoid cultures and showed that β-catenin binds and activates TCF-3 in these tumors (Tejpar *et al.*, 2001). This is in contrast to colon neoplasia, in which β-catenin interacts predominantly with TCF-4. The fact that desmoid tumors show a differential expression of TCF/Lef family members compared to colon cancer could result in the activation of different target genes. In addition, TCF transcription factors are architectural factors, and as such, they may alter transcription in a cellular context-dependent manner, dependent on other factors regulating transcription in the cells. Desmoids and colon tumors originate from different cells, and have a very different *in vivo* behavior and outcome. Altogether, we assumed that genes regulated by TCF-dependent transcriptional activation in mesenchymal desmoid tumors would not necessarily be identical to the target genes found in epithelial tumors.

In this study, we aimed to identify these genes in desmoid tumors. We hypothesized that the target genes would be at least partially responsible for the cell behavior in these tumors and that identification of these genes would give novel insights into how β-catenin modulates fibroblast behavior. Desmoids are benign untransformed lesions that do not carry the later-stage

mutations such as k-ras and p53 that occur during colon cancer progression. Therefore, desmoids are a simpler model for the identification of β-catenin target genes.

Oligonucleotide arrays were used to examine global gene expression patterns in desmoids. A comparison of transcriptional levels between desmoids and normal fascia fibroblasts of the same patients identified 69 genes that appear to be differentially expressed. The insulin-like growth factor-binding protein 6 (IGFBP-6) was identified as a gene that was consistently downregulated in primary desmoid cultures compared to primary fascia cultures. Downregulation of genes by β-catenin is a novel mechanism that has hardly been reported (Fujita et al., 2000). Since IGFPB-6 contained potential β-catenin Tcf-regulatory sites, this gene was chosen for further analysis. IGFBP-6 is a member of a family of six IGF-binding proteins that bind and modulate the actions of the insulin-like growth factors, IGF-I and IGF-II (Bach, 1999). The binding protein is expressed in many cell types, such as fibroblasts, myoblasts, smooth muscle cells, keratinocytes, and osteoblasts. IGFs are potent mitogenic agents, which act predominantly through the IGF-I receptor. IGFBP-6 differs from the other binding proteins because of its 30–100-fold preferential binding affinity for IGF-II over IGF-I (Bach, 1999). Therefore, IGFBP-6 is considered to be

a relatively specific inhibitor of IGF-II actions by sequestering IGF-II and preventing it from binding to the IGF-I receptor. We will show that the IGFBP-6 gene is downregulated in desmoids by stabilized β-catenin, through binding of the TCF complex to two TCF-binding sites in the human IGFBP-6 promoter. Tissue culture experiments further suggest a role for the IGF axis in the proliferation of desmoid tumors.

Results

Expression profiles

To identify downstream genes of β-catenin/TCF in desmoid tumors, microarray analysis was carried out. Using Affymetrix oligonucleotide arrays, gene expression profiles of four primary desmoid and fascia cell cultures were obtained. Results were expressed as the ratio between the values measured in the desmoid samples and those in the fascia samples. Following the selection criteria as described in Materials and methods, a total of 69 genes were identified as differentially expressed in desmoids compared to fascia. Of these 69 genes with significantly altered expression, 33 genes were found to be upregulated and 36 genes downregulated (both ⩾2.5-fold). Table 1 lists the genes that have

Table 1 Differentially expressed genes in desmoids from four patients (Pt1–Pt4) compared to their respective fascia

HUGO	Description	Pt1	Pt2	Pt3	Pt4
(a) Overexpressed genes					
ACTG2	Actin gamma 2	3.3	4.4	15.3	9.2
ADAM19	A disintegrin and metalloproteinase domain 19		2.6	7.6	4.8
AHR	Aryl hydrocarbon receptor	5.1	3.3	3.1	3
ALDH5	Aldehyde dehydrogenase 5	8.6	4.3	3	4.1
ARL7	ADP-ribosylation factor-like 7		4.8	11.5	8.8
CALB2[a]	Calbindin 2	11.1	49.7	10	9
CCND2	Cyclin D2	12.8	12.3	3.7	12.6
CHN1	Chimerin 1	9.5	5	2.6	5.9
CLECSF2	C-type lectin, superfamily member 2	37.4	7.2	12	16.7
CNN1	Calponin 1, basic, smooth muscle	7.6	3.7	−1.6	15.9
	Collagen, type VI, alpha 1				
COL6A2	Collagen, type VI, alpha 2	9.7	4.9	8.3	3.4
	Collagen, type VI, alpha 3				
CSRP2[a]	Cysteine and glycine-rich protein 2		16	7.8	9
CTSK	Cathepsin K	5	3.6	6.7	1.6
GAS1[a]	Growth arrest-specific 1	2.9	1.5	9.6	6.5
HOXB2	Homeo box B2	2.6	1.5	4.2	2.7
HSPA2	Heat shock 70 kDa protein 2	6	5.4	5.9	2.8
IGF2	Insulin-like growth factor 2	3.7	13.3	3.5	1
IGSF4[a]	Immunoglobulin superfamily member 4		15.5	10.8	9.6
	Integrin-linked kinase				
MAB21L1	Mab-21 (*C. elegans*)-like 1	5.2	4.2	9.7	−1.1
MDK	Midkine (neurite growth-promoting factor 2)	10	3.2	4.6	3
MMP3	Matrix metalloproteinase 3	7.5	8.7	64.7	34.9
MYRL2	Myosin-regulatory light chain 2, smooth muscle isoform	3.8	3.2	−2.2	3.1
NRG1	Neuregulin 1		4.6	7.7	2.6
PTK7	PTK7 protein tyrosine kinase 7		18.1	9.6	19.5
SGCD[a]	Sarcoglycan delta	10.1	2.7	2.3	3.4
SLC7A8	Solute carrier family 7 member 8		9.6	9	3.6
SPARCL1	SPARC-like 1 (mast9, hevin)	5.7	1	5.5	5.5
TRO	Trophinin		3	3.8	3.8
WNT5A	Wingless-type MMTV integration site family, member 5A	6.2	3.7	2.4	6
ZIC1	Zic family member 1	14.8	1	5	11.8

Table 1 Continued

HUGO	Description	Pt1	Pt2	Pt3	Pt4
(b) Underexpressed genes					
AGC1	Aggrecan 1	−7.8	−2.9	−9.2	−4.3
ALCAM[a]	Activated leukocyte cell adhesion molecule		−3.5	−5	−8.1
ALDH6	Aldehyde dehydrogenase 6	−7.2	−10.3	−5.8	−1.5
ATF5	Activating transcription factor 5		−6.7	−4.5	−3.2
BENE	BENE protein	−2.5	−8.6	−14.8	−47.9
BRF2	Butyrate response factor 2	−3.1	−1.1	−3	−4.8
CREBL1	cAMP responsive element binding protein-like 1		−7.6	−20.8	−12.8
CRIP1	Cysteine-rich protein 1		−8.4	−8.2	−3.1
CRLF1	Cytokine receptor-like factor 1		−8	−42.6	−5.3
CRYAB	Crystallin alpha B		−5	−11.5	−2.7
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	−14.4	−17.2	−17.4	−2.1
ENG	Endoglin	−5.5	−2.6	−6.2	−1.4
FLNB	Filamin B, beta		−3	−11	−4.5
IFI30	Interferon, gamma-inducible protein 30	−5.5	−6.7	−5.2	−1.2
IGFBP6	Insulin-like growth factor-binding protein 6	−53.7	−4.4	−46.6	−10
ITPR3	Inositol 1,4,5-triphosphate receptor, type 3	−8.2	−1.1	−5.5	−3.55
JUNB	Jun B proto-oncogene	−9.2	−6.2	−2.35	−3.7
	Keratin 18		−4.4	−27.4	1
KRT7	Keratin 7		−15.1	−6.4	−5.4
LGALS3BP	Lectin, galactoside-binding, soluble, 3 binding protein	−6	−6.1	−27.8	3.1
MFGE8	Milk fat globule-EGF factor 8 protein	−2.8	−1.3	−20.1	−3
NID2	Nidogen 2	−5.6	−3	−7.6	1.9
PENK	Proenkephalin	−4	−10	1.1	−5.4
PODXL	Podocalyxin-like		−7.8	−14.1	−14.2
PRG1	Proteoglycan 1, secretory granule	−7.6	−6	−11.8	−3.8
PTGIS	Prostaglandin I2 synthase		−17.5	−48.5	−12.7
PTX3	Pentaxin-related gene	−8.5	−4.7	−12.2	−2.5
QSCN6	Quiescin Q6	−2.7	−1.6	−3	−2.7
SDF1	Stromal cell-derived factor 1	−5.8	−7.3	−1	−13.5
SEMA3C	Semaphorin 3C	−1.4	−3	−3.3	−3.3
	Serine (or cysteine) proteinase inhibitor member 2				
SFRP1	Secreted frizzled-related protein 1		−14.9	−5.5	−12
TNA	Tetranectin	−30.3	−2	−7.3	−3.9
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	−4.5	−3.2	−6.2	1.7
TNFRSF11B	Tumor necrosis factor receptor superfamily, 11b		−28.7	−9.7	−10.2
TRH	Thyrotropin-releasing hormone	−27.9	−7.5	−7	−16.3

Values represent the relative expression ratio of genes in four desmoids against the corresponding fascia. Only genes that have an absolute ratio of minimum 2.5 in at least three of the four patients and that are significantly (>4 ×) above the background are selected. [a]Sequences that are not mapped on the Ensembl genome assembly. For Pt1, a 6.8 K gene array was used, replaced by a new 19 K gene array for Pt 2, 3, and 4; hence the missing data for some genes in Pt1

shown a ⩾2.5-fold increase or decrease in expression level in at least three of four independent experiments.

Confirmation of differentially expressed genes

To test the general reliability of the microarray data, semi-quantitative reverse transcription (RT)–PCR (TaqMan) analysis was performed on a selection of genes of Table 1. Although the absolute magnitude of the relative expression level determined by RT–PCR differed sometimes from that measured on the arrays, the direction of change in expression was consistent between the different techniques (Figure 1).

Expression of colon-cancer β-catenin/TCF target genes in desmoids

In colon cancer, target genes of β-catenin/TCF-mediated transcription comprise, among others, the genes encoding for cyclin D1, c-myc, fra-1, c-jun, MMP7, and PPAR delta. Since none of these were present in Table 1, expression profiles of these genes

were generated in four desmoid and fascia samples using quantitative RT–PCR (TaqMan). Of these six genes, only MMP7 was upregulated in all desmoids compared to the controls (mean 33-fold) (Figure 2). C-myc, c-jun, and PPAR delta were not differentially expressed. For cyclin D1 and fra-1, the results were inconsistent. Both genes were upregulated in only half of the patients.

Gene regulation bioinformatics

Hypothesizing that a number of the selected up- and downregulated genes (see Table 1) can be real target genes of the β-catenin/TCF complex, we investigated the putative TCF-binding sites upstream of the coding sequence and their flanking regions, in order to unravel some of the regulatory principles of this complex in desmoid tumors. Especially, differences in the promoters between upregulated and downregulated target genes are interesting, because our results suggest that β-catenin might also downregulate target genes (see further). Further background and methods are available at http://www.esat.kuleuven.ac.be/~dna/biol/softwar-
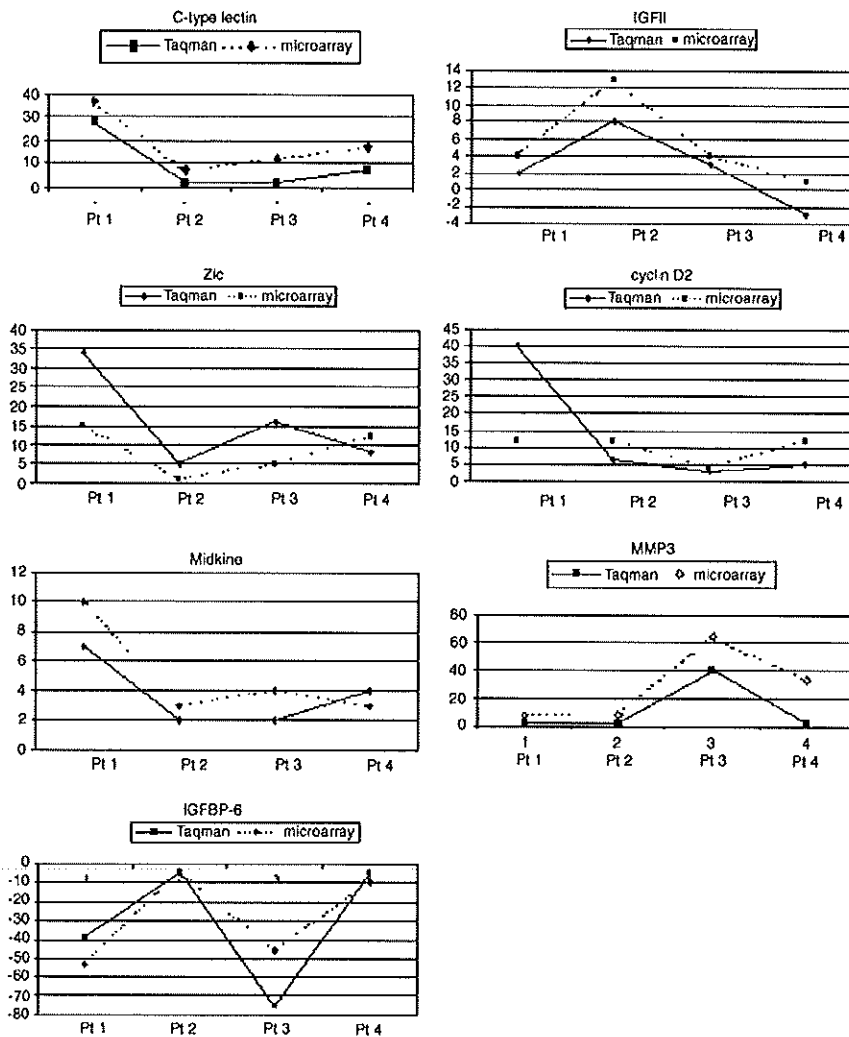
Figure 1 Comparison of expression ratios obtained by Affymetrix microarrays and quantitative RT–PCR (Taqman) of seven selected genes in four patients (Pt). The black boxes on the full line represent the ratios obtained by RT–PCR, the black boxes on the striped line represent the ratios obtained by microarray

e.html. Briefly, we found that the number of genes within our sets that have a TCF site within the first 2000 bp upstream of the CDS was not greater than expected. In the set of upregulated genes, using 100 bp flanking the first TCF site, we found that the binding sites for CDXA, OCT1, GATA2, OCT1, EN1, and STAT5A were significantly over-represented (SIG > 4). For the downregulated genes, the over-represented patterns were binding sites for GEN-INI2, GEN-INI, IK-2, STAT5A, GEN-INI3, and ISRE.

Downregulation of IGFBP-6

IGFBP-6 was identified as a gene that was differentially expressed and downregulated in all desmoids tested, compared to fascia.

Confirmation at the RNA level

Northern blot analysis was used to validate the results of IGFBP-6 in the microarray experiments. A major band of 0.952 kb was detected in all fascia samples tested, while the desmoids samples showed no or only a weak staining (Figure 3). The down-regulation of IGFBP-6 mRNA in desmoid cells compared to fascia cells further confirmed by quantitative RT-PCR (Figure 1).

Confirmation at the protein level

To confirm the expression of IGFBP-6 mRNA at the protein level, Western blot analysis was performed with desmoid and fascia conditioned medium (CM). Using an anti-IGFBP-6 antibody, an apporximately 32-kDa band was seen only in the CM of fascia (Figure 4). The size of the band was consistent with that previously described for IGFBP-6 (Kim et al., 2002). None of the desmoid CM samples demonstrated any detectable bands. These results confirmed the differential expression of IGFBP-6 in desmoid and fascia cultures.

expression of colon cancer target genes in fascia/desmoid
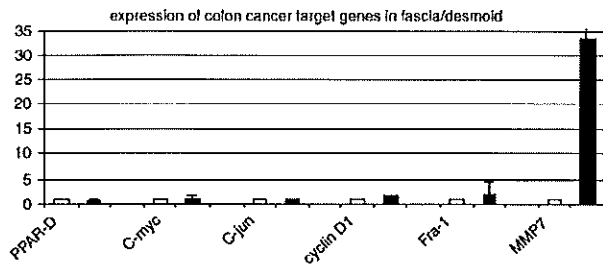
Figure 2 Comparison of expression ratios between desmoid and fascia obtained by quantitative RT–PCR (Taqman) of six selected known β-cat target genes in colon cancer genes in four patients (Pt). Median values of the results obtained in four patients are shown. The black bars represent desmoid samples, the white bars fascia samples
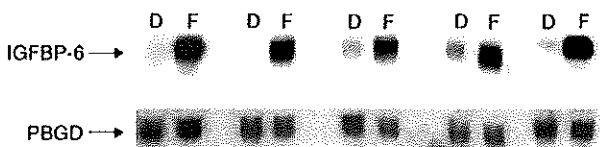

Figure 3 Northern Blot analysis of IGFBP-6 RNA in five desmoid tumors (D) compared to the control fascias (F) from the same patients. Total RNA (15 μg) from the cells was subjected to electrophoresis, transferred to nitrocellulose membranes, and hybridized with a human IGFBP-6 probe. To control the differences in total RNA, the blot was stripped and hybridized with a probe for the human PBGD gene

Figure 4 Western blotting analysis of IGFBP-6 secretion by desmoid and fascia cells. Conditioned media of five desmoids (D) and matched fascia (F) primary cultures were collected and concentrated, as described in Materials and methods. Immunoblot analysis was performed, by probing with an anti-human IGFBP-6 polyclonal antibody. The volumes of the media loaded on the gel were adjusted for equivalent cell numbers. Migration of the 30 kDa molecular weight marker is shown on the left

β-catenin regulates the expression of IGFBP-6

To determine whether the repression of the IGFBP-6 promoter was mediated by β-catenin, the IGFBP-6 promoter pGL3 luciferase construct was cotransfected into CHO cells with different doses of β-catenin mutant plasmids and luciferase analysis was carried out. Figure 5 shows that the IGFBP-6 luciferase reporter was repressed in response to S33 mutant β-catenin, in a dose-dependent manner. Interestingly, the N-terminus of β-catenin appeared to be important in mediating this repression, as the delta N-89 β-catenin mutant was unable to repress the IGFBP-6 promoter, whereas other forms of stabilized beta cat such as S45 repressed the promotor. These results suggested that IGFBP-6 can be regulated by β-catenin and might be a target of the Wnt signaling pathway.
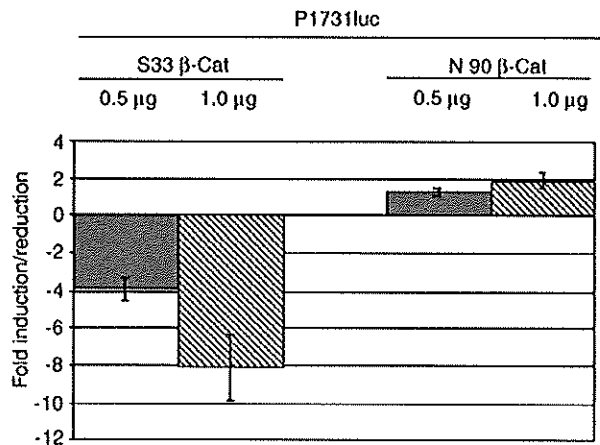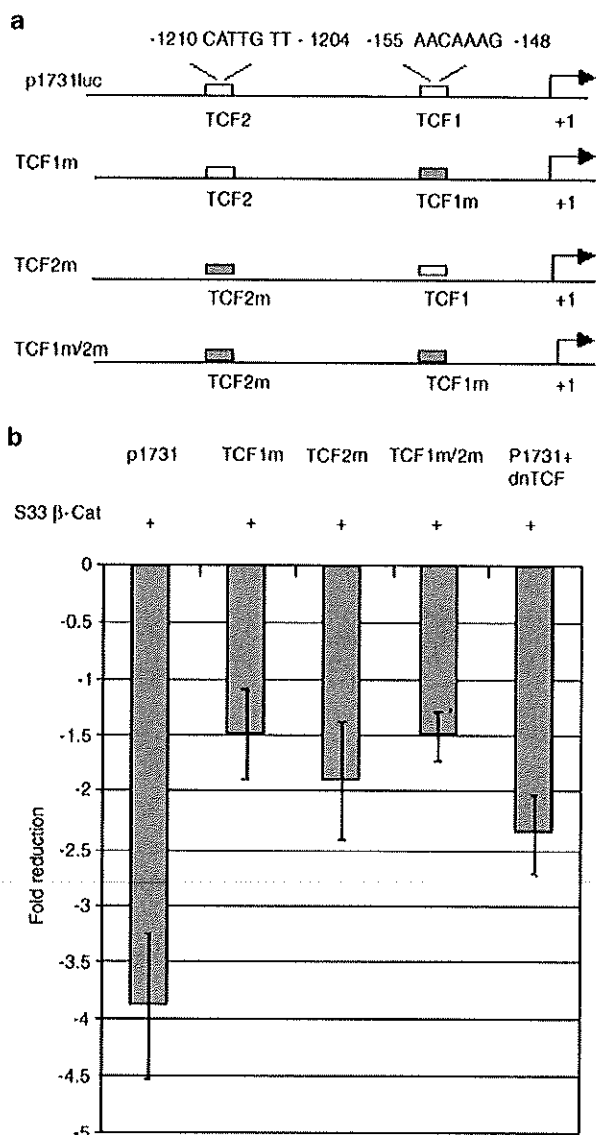

Figure 5 IGFBP-6 promoter luciferase assays. Luciferase transfection assays with the wild-type IGFBP-6 promoter (p1731luc) in CHO cells were performed as described in Materials and methods. The IGFBP-6 promoter is repressed dose dependently upon cotransfection with mutant S33 β-catenin. Transfection with a delta N-90 deletion β-catenin plasmid did not repress the IGFBP-6 promoter

Role of the TCF sites in the downregulation of the IGFBP-6 promoter by β-catenin

DNA-binding factors of the TCF/Lef family are known to interact with β-catenin and alter the transcription of downstream genes. To evaluate whether the β-catenin/TCF signal transduction pathway regulated IGFBP-6 expression, we analysed the human IGFBP-6 promoter sequence for the presence of TCF-binding elements. Two potential TCF-binding sites in a head-to-tail configuration were identified, 155 (TCF1) and 1210 (TCF2) bp upstream from the transcriptional start site of IGFBP-6, matching the consensus for the TCF-binding sequence (Roose and Clevers, 1999) (Figure 6a). To investigate the hypothesis that these two motifs were involved in the transcriptional repression, 2 bp point mutations were introduced into the TCF-binding sites that should render these sites inactive (Brannon et al., 1997).

Next, mutated (TCF1m, TCF2m, and TCF1m/2m) IGFBP-6 promoter constructs were cotransfected with the S33 β-catenin mutant. Mutations in either TCF1 or TCF2 significantly reduced β-catenin-dependent suppression from approximately fourfold to 1.5-fold (Figure 6b). Knockout of both sites did not significantly affect the responsiveness of the IGFBP-6 promoter to β-catenin to any higher degree than the single-site mutations. These results imply that the two TCF-binding sites are involved in suppression of IGFBP-6 transcription.

Cotransfection of a dominant-negative TCF construct, which binds the TCF sites but cannot bind β-catenin, with the wild-type promotor construct and S33 β-catenin significantly reduced the repression mediated by β-catenin, again indicating a role for the TCF/beta complex in the repression of this promotor.

a

b

Figure 6 (a) Schematic representation of the IGFBP-6 promoter. The promoter of IGFBP-6 contains two putative TCF motifs (boxes at −1210 and −155). The IGFBP-6 promoter luciferase reporter construct (p1731 luc) was mutated (black box) at only one of the two TCF-binding sites (TCF1m, and TCF2m), leaving the other intact, or at both sites (TCF1m/2m). (b) The wild-type and mutant IGFBP-6 promoter constructs were cotransfected with 0.5 μg S33 β-catenin. Mutations in either or both TCF sites in the promoter resulted in a reduction of β-catenin-dependent repression. Cotransfection of a dominant-negative TCF construct, with the wild-type promotor construct and S33 β-catenin significantly reducing the repression mediated by β-catenin

Electromobility shift assays (EMSA) experiments were performed to confirm a direct mode of interaction between the TCF complex and the promoter. The observed supershift obtained with a TCF-3/TCF-4 antibody showed that there is specific binding of the TCF protein to both TCF recognition sites in the IGFBP-6 promoter (Figure 7).

Additional DNA protein pulldown assays using the TCF sites as bait were able to pull down the β-catenin
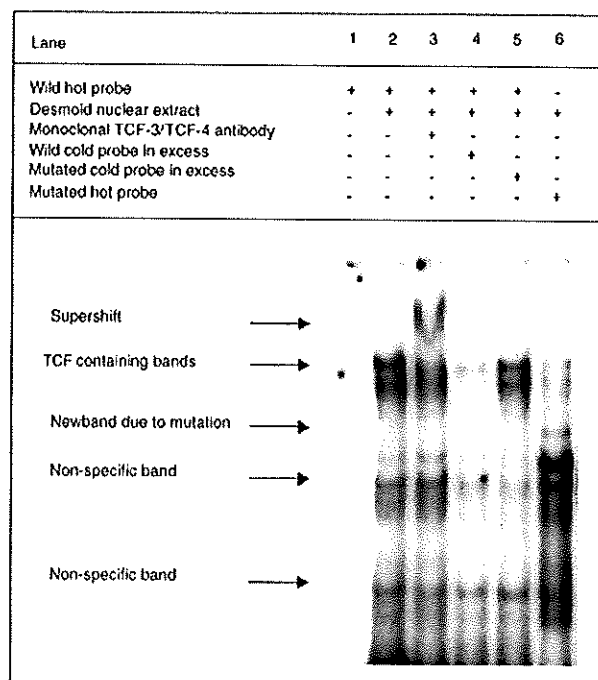

Figure 7 Electromobility shift assays. The first lane shows the TCF2 probe without any nuclear extract. The second lane shows the shift by adding the desmoid nuclear extract. In the third lane, the supershift is obvious after incubation with a monoclonal anti-TCF-3/TCF-4 antibody; the first two bands are weakened, suggesting the presence of TCF in the bands. The fourth lane demonstrates the effect of excess wild cold probe, reducing the intensity of all bands, including the TCF bands. In the fifth lane, the excess mutated cold probe is unable to compete with the wild probe in binding to TCF. The last band shows that the mutated hot probe binding capability to TCF is dramatically decreased
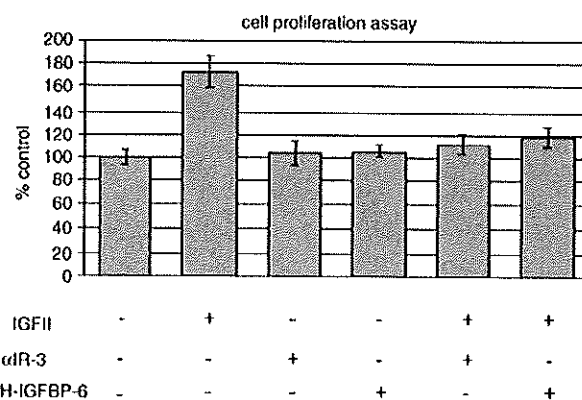
Figure 8 Cell proliferation assays. The effect of IGF-II (100 ng/ml), rH-IGFBP-6 (1000 ng/ml) and αIR-3 (1 μg/ml) on proliferation of desmoid cells was examined by counting the cell numbers, by coulter counter after 72 h. The results are expressed as a percentage of the untreated control. Values are the means ± s.d. of triplicate wells. The experiment was repeated twice with similar results

protein, providing further evidence of downregulation of gene expression by a complex containing Tcf and β-catenin protein (data not shown).
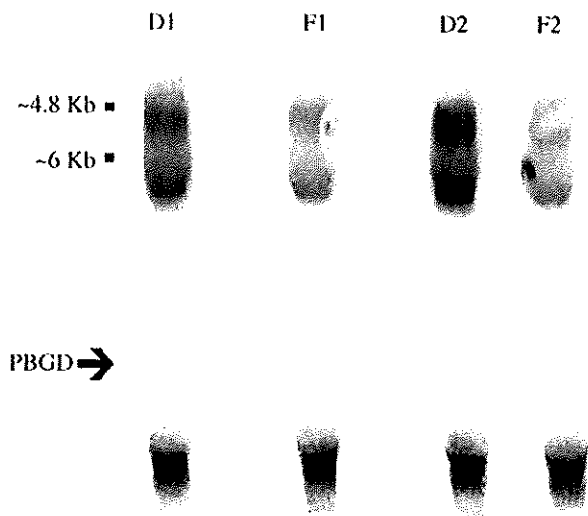
Figure 9 Northern Blot analysis of IGF2 RNA in two desmoid tumors (D) compared to the control fascias (F) shows approximately four times upregulation of IGF 2 RNA in desmoids. Note that two transcripts of IGF2 are detected, corresponding to transcripts from the P3 (6 kb) promoter and transcript from the P4 (4.8 kb) promoter

Growth curves

IGFBP-6 is a relatively specific inhibitor of IGF-II action (Bach, 1999) and changes in the levels of IGFBP-6 can influence the actions of IGF-II. Cell proliferation assays were performed to assess the biological significance of IGFBP-6 downregulation by β-catenin in desmoids (Figure 8). First, we tested whether IGF-II could act as a mitogen in desmoid cells by performing proliferation assays with desmoid cells treated with exogenous IGF-II. The cell number increased to $173 \pm 14\%$ of controls when desmoid cells were incubated with IGF-II (100 ng/ml) for 72 h. To determine the effect of IGFBP-6 on IGF-II-stimulated proliferation, IGF-II was coincubated with rH-IGFBP-6 (1000 ng/ml). As shown in Figure 8, coincubation of IGFBP-6 with IGF-II inhibited proliferation to $119 \pm 9\%$ of control. Addition of IGFBP-6 alone had no effect on basal proliferation.

To show that IGF-II actions were mediated by the IGF-I receptor, IGF-II was coincubated with a monoclonal antibody blocking the IGF-I receptor (αIR-3), which blocks the action as well as binding of IGFs to the IGF-I receptor (Cullen *et al.*, 1992). At 1 μg/ml, αIR-3 almost completely abolished IGF-II-stimulated proliferation.

The basal levels of IGFII RNA in desmoids compared to fascia were found to be moderately upregulated, approximately $4 \times$, when assessed by Northern blot (Figure 9), confirming the findings of the Affymetrix chip (see Table 1). However, an RIA assay for IGFII protein (data not shown) showed no difference between desmoid and fascia at the protein level.

These results demonstrate that IGFBP-6 can inhibit the actions of IGF-II in desmoid cultures, and support

the hypothesis that the downregulation of IGFBP-6 in desmoid tumors is instrumental in the proliferation stimulated by IGF-II.

Discussion

Although β-catenin is identified as a key molecule in desmoids, the molecular fingerprint of desmoids still remains largely unclear. Differential gene expression profiles were generated with oligonucleotide arrays. The results showed that multiple genes are differentially expressed in desmoids, 33 genes were upregulated 2.5-fold or higher and 36 genes were downregulated 2.5-fold or higher at the mRNA level. No obvious differences in the expression patterns between APC- or β-catenin-mutated tumors were observed, although our set of tumors may have been too small to detect subtle differences *if* present.

Many of the differentially expressed genes encode components of pathways that have been implicated in cancer, such as overexpression of genes involved in cell cycle regulation (cyclin D2), proteolysis of the extracellular matrix (MMP3, MMP7), and growth stimulation (IGF-II, Midkine). Genes that inhibit some of these processes are repressed, for example IGFBP-6 (inhibitor of IGF-II). Quantitative RT-PCR on a subset of differentially expressed genes confirmed the array results (Figure 1), suggesting that they were reliable and that the genes in Table 1 could be considered as potential β-catenin-regulated genes.

The results also showed that genes regulated by β-catenin-mediated TCF-dependent transcription in mesenchymal desmoid tumors are not necessarily identical to the target genes found in epithelial tumors. Of the β-catenin target genes identified in colon cancer, only MMP7 was upregulated in all desmoids tested. Cyclin D1 and fra-1 were upregulated in 50% of the desmoid samples. The results for cyclin D1 expression in desmoids are comparable to those found by Saito *et al.* (2001). No differential expression was observed for c-jun, c-myc, and PPAR delta. The fact that PPAR delta was not overexpressed in desmoids confirmed the data from Poon *et al.* (2000).

These results are not surprising because we showed, previously, that only TCF-3 is consistently expressed in all desmoids, in contrast with colon cancer, where it was shown that mainly TCF-4 was expressed (He *et al.*, 1998), and, in contrast to pilomatricomas, in which Lef-1 is expressed by the tumor cells (Gat *et al.*, 1998). Thus, the differences in cell type and in TCF expression may be responsible for the transcription of different targets of Wnt-β-catenin signaling. Another explanation for the activation of different target genes in desmoids versus colon cancer could be a different level of nuclear β-catenin. Indeed, desmoid extracts express significantly lower levels of β-catenin protein in Western blot than colon carcinoma cell lines (results not shown). According to the 'just-right signaling' model of Albuquerque *et al.* (2000), a subtle change in the level

of signaling-competent β-catenin may have a drastic impact on gene activation.

Next, we wanted to investigate the possibility that the β-catenin/TCF complex might downregulate target genes in a direct manner. The expression of IGFBP-6 was further analysed in desmoid tumors because IGFBP-6 was consistently downregulated by all techniques in all patients, and because potential downregulation of genes by β-catenin/TCF complexes has hardly been studied.

Promoter reporter assays demonstrated that mutant S33 β-catenin suppressed IGFBP-6 promoter activity. When the promoter contained a two base substitution in either or both potential TCF-binding sites, the suppression by β-catenin was significantly decreased, suggesting the involvement of the TCF sites. Cotransfection of dominant-negative TCF constructs with S33 β-catenin significantly inhibited the downregulation of the promotor by β-catenin. An electrophoretic mobility shift assay using the two potential TCF-binding sequences revealed the interaction of the candidate sequences with the TCF complex, and DNA protein pulldown identified β-catenin in the DNA–TCF complex. Taken together, these results suggest that the β-catenin/TCF complex might be directly involved in the downregulation of IGFBP-6.

The β-catenin/TCF complex is mainly known to activate target genes. In the simplest model of Wnt/β-catenin signaling, the TCF/Lef transcription factors become transcriptional activators of target genes upon binding of β-catenin and coactivators (Cadigan and Nusse, 1997). In the absence of nuclear β-catenin, TCF/Lef together with corepressors can repress transcription (Roose et al., 1998). It is, however, becoming clear that the β-catenin/TCF complex does not regulate all target genes in the same way and that, depending on the promoter of the target gene and cell type, they can recruit different cofactors (Hecht and Kemler, 2000).

While β-catenin has been mainly associated with transcriptional activation, both our results and that from two studies in mice (Fujita et al., 2000; Kielman et al., 2002), suggest that β-catenin might also downregulate target genes. How β-catenin contributes to transcriptional repression is not yet clear. One possible model is the binding of promoter-specific cofactors to the β-catenin/TCF complex, which somehow enhances repression. It was previously shown that β-catenin not only binds coactivators, but can also bind a co-repressor, Reptin52, and that the repressive effect of Reptin52 depends on the presence of β-catenin in the β-catenin/TCF transcription complex (Bauer et al., 2000). Another possibility is a cooperative interaction between the β-catenin/TCF complex and a nearby transcription factor, because the architectural TCF proteins can cooperate with the factors bound at nearby sites, which determine the mode of regulation. We therefore analysed the sequences around the promoter-proximal TCF sites in the downregulated genes, and detected some over-represented transcription factor-binding sites (see supplementary data). In the IGFBP-6 promoter, a

putative regulatory module might consist of a TCF site, together with a STAT5A and an IK-2 (or Ikaros)-binding site. Another explanation for the downregulation of β-catenin target genes could be the presence of alternative isoforms of TCF transcription factors that, instead of activating the promoter of target genes in the presence of β-catenin, repress transcription. So far, no alternative splice forms of human TCF-3 have been described, however.

Altered regulation of the IGF system has increasingly been linked to malignancy (Cui et al., 2002). In colon cancer, where mutations in APC or β-catenin are the earliest genetic alterations, the IGF system may play an important role in proliferation, as the single most-expressed gene in colorectal cancer relative to normal colonic mucosa is IGF-II (Zhang et al., 1997). As IGFBP-6 is considered to be a specific inhibitor, changes in IGFBP-6 secretion may be biologically important in modulating the availability of IGF-II for IGF receptors. In desmoid, IGF-II RNA levels were found to be moderatly elevated ($\times 4$), whereas the IGFII protein itself was not elevated in comparison to fascia. This is a known finding in the IGFII system, in which not all IGFII RNAs are necessarily translated (de Moor et al., 1995). Thus, desmoids express normal levels of IGFII protein, whereas IGFBP6 is significantly downregulated. In colorectal cancers, IGFBP6 levels were found to be normal (data not shown). The net effect of these inverse variations might be an increased bioavailability of IGF-II in both tumor types. Similar to the results of in vitro studies with other cell types, the cell proliferation assays in primary desmoid cultures showed that rH-IGFBP-6 was able to inhibit the IGF-II-induced proliferation. Taken together, it would seem that the desmoid cells are sensitive to exogenous IGF-II and that downregulation of the IGFBP-6 gene by β-catenin is instrumental in the IGF-II-stimulated proliferation.

In conclusion, expression profiles of desmoid tumors showed that multiple genes are differentially expressed. Recently, it was shown that β-catenin signaling is involved in normal and pathological cutaneous wound healing (Cheon et al., 2002). Owing to the cytological similarity between desmoid cells and fibroblasts in the proliferative phase of wound healing, the differentially expressed genes may not only represent new targets for therapeutic intervention for desmoid tumors, but also for other fibroblast proliferations.

This study has also shown that the repressed expression of IGFBP-6 in desmoid tumors is due to the fact that the IGFBP-6 promoter responds transcriptionally to the β-catenin/TCF complex. Besides the identification of a downregulated β-catenin gene MCP-3 in mice (Fujita et al., 2000), and the recent suggestion of β-catenin downregulated genes in ES cells (Kielman et al., 2002), this is the first report of a β-catenin downregulated target gene in human cancer. How the β-catenin/TCF complex can downregulate target genes has not been resolved yet, and is presently a subject of investigation.

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Materials and methods

Cell lines and materials

The cell line CHO was obtained from the American Type Tissue Culture Collection and cultured in Dulbecco's modified medium (DMEM) (Invitrogen, Merelbeke, Belgium), supplemented with 10% fetal calf serum (FCS) (Hyclone, Erebodegem-Aalst, Belgium). The αIR-3-blocking IGF-I receptor monoclonal antibody was purchased from Oncogene Research Products (Darmstadt, Germany); BSA from Sigma-Aldrich; recombinant human IGFBP-6 (rH-IGFBP-6) from Austral Biologicals (San Ramon, CA, USA); anti-IGFBP-6 antibody (sc-6007) from Santa Cruz (CA, USA); TCF3/4 monoclonal antibody from Exalpha Biologicals, Inc. (Boston, MA, USA).

Samples and cell cultures

Primary cell cultures of four desmoid tumors were derived by collagenase treatment of tissue biopsies and grown in DMEM supplemented with 10% FCS. Normal fascia tissue at a safe margin from the resection sides was also obtained and processed in an identical manner. The cultures were divided when confluent and all studies were performed using cultures in passage two. For the array experiments, four desmoids, two APC and two β-catenin-mutated tumors, were used. To confirm the tumoral origin of the cultured cells, all primary tumor cultures were examined for the presence of nuclear β-catenin by immunohistochemistry and for transcriptional TCF-dependent activation by TOP/FOP transfection experiments. Only tumor cultures demonstrating clear nuclear β-catenin and a significant high TOP/FOP ratio were used for expression-profiling experiments. In parallel, fascia primary cultures were checked for the absence of nuclear β-catenin and transcriptional TCF-dependent activation.

RNA extraction

Total RNA was extracted from primary desmoid and fascia cultures using the RNeasy kit (Qiagen), following the manufacturer's instructions.

Oligonucleotide arrays

Using the Affymetrix HuGeneFL Arrays, mRNA expression profiles were made from primary desmoids and fascia cultures from the same patients. For the first experiment, a 6.8 K gene array was used, next a new 19 K gene array replaced the old type and, for this reason, subsequent cases were analysed on the 19 K chip. Briefly, double-stranded cDNA was synthesized from 20 μg of total RNA with oligo(dT)$_{24}$ T7 primer, amplified with T7 RNA polymerase and hybridized to the oligonucleotide array according to the manufacturer's instructions. After washing, the remaining biotinylated RNA was stained and scanned using a GeneArray scanner (Affymetrix), and image analysis was performed with Genechip 4.0 software (Affymetrix).

For a gene to be selected as differentially expressed, it had to be expressed at least 2.5-fold higher or lower in the desmoid samples compared to the fascia samples, and with a minimum difference in hybridization signal of 200. Where expression was below the baseline, it was determined to be absent and set at 50, the background level.

RT–PCR (TaqMan)

Quantitative PCR was carried out by ABI PRISM 7700® Sequence Detection Systems (Applied Biosystems). After RNA extraction, as described above, cDNA was synthesized using random primers (Amersham Pharmacia) and SuperScript II (Life Technologies, Inc.). Probes and primers were designed by Primer Express 1.0 (Applied Biosystems). Sequences of the primers and probes are available upon request. Using the TaqMan PCR kit (Eurogentec), PCR protocol was carried out as recommended by Applied Biosystems. Standard curves for targets and the housekeeping control gene PBGD (Porphobilinogen Deaminase) were drawn by Excel (Microsoft) upon the Ct (threshold cycle) values, and the relative concentrations of the standards and the relative concentrations for desmoid and fascia samples were calculated from the detected Ct values and the equation of the curves. Values obtained for targets were divided by the values of PBGD to normalize for differences in reverse transcription.

Genomic contamination of the samples was checked by NAC (No Amplification Control) samples, which did not contain reverse transcriptase enzyme during the cDNA preparation.

Northern blot

For Northern Blotting, 15 μg of total RNA was denatured in a MOPS/formaldehyde/formamide buffer and run on a 1% agarose gel. The RNA was transferred onto Hybond-N nylon membranes (Amersham Pharmacia Biotech) overnight by capillary force. Specific cDNA sequences of IGFBP6, IGFII and PBGD were amplified by RT–PCR, isolated and used as probes. After prehybridization, hybridization was carried out overnight at 68°C in an ExpressHyb hybridization solution (Clontech) with a 32P-labeled probe. Membranes were washed during 1 h at 42°C with a 2 × SSC, 0.1% SDS solution and during 1 h at 62°C with a 0.1 × SSC, 0.1% SDS solution. After autoradiography, all Northern blots were stripped and hybridized with a cDNA probe for PBGD to control for RNA loading and transfer efficiency.

Plasmids

The delta N89 β-catenin plasmid was obtained from Polakis. This CMV-neo-bam vector lacks the first 89 codons of the β-catenin gene crucial for protein degradation. Expression vector for mutant β-catenin, S33-β-catenin (codon 33 substitution of tyrosine for serine) was obtained from Vogelstein. The dominant-negative TCF construct is able to bind DNA, but lacks the β-catenin transactivation sites, thus competing with and inhibiting wild-type TCF promotor regulation. This construct was obtained from Vogelstein.

The p173lluc construct, containing the 1.7 kb human insulin-like growth factor-binding protein (IGFBP)-6 gene 5'-flanking region (Accession No. AF297519), was a kind gift from Donna Strong (Loma Linda, CA, USA).

Site-directed mutagenesis on the p173lluc (IGFBP-6 promoter) vector was performed with the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The mismatched oligonucleotides used to eliminate TCF sites 1 and 2 were as follows:

- TCF1m: 5'-CCGAGATTCCCGGGGCCAAAGCAAGA AAAATCAGAGC-3'
- TCF2m: 5'-GGCCTTGCTGACATTGGCGCTTGGGGC CC-3'

to give the mutated vectors TCF1m, TCF2m, and TCF1m/2m. All constructs were sequenced to confirm that only the intended point mutations were introduced.

Transfections

CHO cells were plated into six-well plates $(1 \times 10^5$ cells/well) in DMEM supplemented with 10% FCS and grown to 75% confluence. Next, cells were transiently transfected using Fugene (Roche Molecular Biochemicals) following the manufacturer's instructions. Total DNA concentrations were kept constant with empty vector DNA. At 30 h after transfection, the cells were harvested and luciferase and β-galactosidase (to control transfection efficiency) assays were carried out as specified by the manufacturer (Promega). Transfections were carried out in triplicate and the means±s.d. of at least three independent experiments are presented.

Western blot

IGFBP-6 protein levels in the conditioned media (CM) of desmoids and fascia cultures were determined by Western blot. CM from 1×10^6 cells was collected after 48 h of serum deprivation and centrifuged at 1000 g for 10 min to remove debris. Protease inhibitors were added to prevent protease activity and the CM was acidified with in glacial acetic acid (1 M) to separate the IGFBPs from endogenous IGFs (Roghani et al., 1991). The CM were concentrated by ultrafiltration in centricon-10 filters, lyophilized and reconstituted in laemmli buffer under nonreducing conditions (Singh et al., 1994), obtaining a 20-fold concentration. The concentrated samples were heated at 95°C for 4 min before samples were applied to a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE 12%) at 35 mA. The size-fractionated proteins were electroblotted onto polyvinylidene fluoride (PVDF; Millipore) membranes for 2 h at 200 mA.

The membranes were blocked with tris-buffered saline (TBS) with milk powder, 5% overnight at 4°C. The membranes were washed three times for 5 min with TBS with Tween 0.1% (TBS-T), and then incubated with an anti IGFBP-6 antibody (1:500 dilution) for 2 h at 20°C with gentle shaking. The membranes were washed five times for 5 min in TBS-T. After incubation with a second anti-goat antibody (1:2000) coupled to horseradish peroxidase (Prosan), the membranes were washed as above. Finally, immunoreactive bands were detected by ECL according to the manufacturer's instructions (Amersham).

Electromobility shift assay (EMSA)

Assays were performed as described previously with minor modifications. As TCF probes, we used double-stranded oligos containing the TCF-binding sites of IGFBP-6 promoter, with a flanking region of 11–15 base pairs. Mutant TCF oligos with two mutated base pairs in the TCF-binding site were used as controls. Nuclear extracts were prepared from desmoid samples by TransFactor Extraction kit from Clontech. The binding reaction mixture contained 3 μg nuclear protein, 250 ng poly(dI-dC) (Amersham Pharmacia) in 25 μl binding buffer (60 mmol/l KCl, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 10% glycerol). A measure of 1.5 μg of monoclonal anti-TCF-3/4 (Exalpha), monoclonal anti-β-catenin (Transduction Laboratories, BD Biosciences) and control antibodies were added to the samples prior to the probes and, after 20 min incubation at room temperature, ~ 8000 c.p.m. probe was added and samples were incubated for another 20 min. The samples were subsequently subjected to nondenaturing polyacrylamide gel electrophoresis, on 6% gels for 5 h in 0.25 × TBE. Sequences of the probes are available upon request.

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DNA–protein pulldown assay

Desmoid nuclear extracts were prepared by a TransFactor Extraction Kit (Clontech). A volume of 20 μl (2 μg/μl) of the nuclear extract was precleared with 35 μl of streptavidin agarose beads (Sigma) at 4°C for 1 h on rotary shaker at low speed. The sample was centrifuged at × 2000 r.p.m., 4°C for 2 min. The supernatant was moved to a new tube and incubated with 30 pmol biotinylated double-stranded Tcf-1 probe, 10 μg Poly dI-dC (Amersham pharmacia) and 1 μl of 0.1 M ZnSO₄ for 3 h at 4°C on a rotary shaker at low speed. Then, 35 μl of streptavidin agarose beads was added and incubated for one more hour. Beads were collected by centrifugation at 2000 r.p.m., 4°C for 2 min. Then, they were washed × 4 by 1 ml ice-cold lysis buffer (20 mM Tris pH = 7.5, 2 mM EDTA, 150 mM NaCl, 0.5% NP40, 50 mM NaF, and complete protease inhibitor cocktail mini-tablet, Roche). The beads were run on 4–12% Nupage Bis-Tris SDS–PAGE gel and transferred to a PVDF membrane (Invitrogen). Western blot was performed by a WesternBreeze™ Chemiluminescent Detection Kit (anti-Mouse) (Invitrogen), as explained by the manufacturer. Monoclonal anti β-catenin mouse antibody (Transduction Laboratories) was used as the first antibody.

Growth curves

For the cell-proliferation studies, cells were plated in six-well plates (Iwaki microplates) in DMEM supplemented with 10% FCS at a density of 3×10^4 cells/well and allowed to attach overnight. After washing twice with phosphate-buffered saline (PBS), the media were changed to serum-free media and left overnight. The next day, cells were incubated in serum-free medium/0.05% BSA with or without IGF-II, and/or rH-IGFBP-6, and/or αIR-3 at 37°C. At the indicated number of days, cells were trypsinized and, after neutralization with DMEM/10% FCS, the cell number in each well was counted with a coulter counter. All experiments were performed twice in triplicate. Each data point is the mean±s.d. A representative result of two different experiments is shown.

Statistics

The means and standard deviations (SD) were determined for each transfection condition, and compared using two-way t-test.

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