FGF-20 and DKK1 are transcriptional targets of β-catenin and FGF-20 is implicated in cancer and development

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β-catenin is the major effector of the canonical Wnt signaling pathway. Mutations in components of the pathway that stabilize β-catenin result in augmented gene transcription and play a major role in many human cancers. We employed microarrays to identify transcriptional targets of deregulated β-catenin in a human epithelial cell line (293) engineered to produce mutant β-catenin and in ovarian endometrioid adenocarcinomas characterized with respect to mutations affecting the Wnt/β-catenin pathway. Two genes strongly induced in both systems—FGF20 and DKK1—were studied in detail. Elevated levels of FGF20 RNA were also observed in adenomas from mice carrying the Apc^Min allele. Both XFGF20 and Xdkk-1 are expressed early in Xenopus embryogenesis under the control of the Wnt signaling pathway. Furthermore, FGF20 and DKK1 appear to be direct targets for β-catenin/TCF transcriptional regulation via LEF/TCF-binding sites. Finally, by using small inhibitory RNAs specific for FGF20, we show that continued expression of FGF20 is necessary for maintenance of the anchorage-independent growth state in RK3E cells transformed by β-catenin, implying that FGF-20 may be a critical element in oncogenesis induced by the Wnt signaling pathway.

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Introduction

Wnt proteins are secreted glycoproteins that bind and activate two classes of co-receptors, LDL-related proteins (LRPs) and members of the Frizzled protein family. Signaling initiated by Wnts and their receptors controls a wide variety of cell processes, including cell fate specification, differentiation, migration, and polarity (reviewed in Peifer and Polakis, 2000). β-Catenin is the major effector of the canonical Wnt signaling pathway. In the absence of Wnt, cytosolic β-catenin forms a complex with Axin and adenomatous polyposis coli (APC) proteins, and is rapidly degraded by the ubiquitination–proteosome system. Wnt signaling inactivates the β-catenin destruction complex, so that β-catenin is stabilized, accumulates in the cytoplasm and nucleus, and forms heterodimers with the DNA-binding factors belonging to the LEF/TCF family (reviewed in Huelsken and Behrens, 2002). As a result, significant changes occur in the gene expression program (reviewed in Hecht and Kemler, 2000; Miller *et al*, 2001). In addition, Wnt signaling can be antagonized extracellularly by secreted factors such as Wnt inhibitory factor-1 (WIF-1), Cerberus, members of the Dickkopf (DKK) family, and soluble Frizzled-related proteins (sFRP) (Kawano and Kypta, 2003).

Genetic alterations that stabilize β-catenin are found in tumors in mice and humans. The most commonly observed genetic alterations involve either the loss of *APC* or *Axin* or mutations that affect the amino-terminus of β-catenin, all occurring in a mutually exclusive manner, and the most commonly affected organs are the colon, liver, skin, stomach, ovaries, pancreas, and prostate. For example, loss of *APC* occurs in 70–80% of human colorectal cancers (reviewed in Bienz and Clevers, 2000) and mutations affecting β-catenin are found in about half of the remaining tumors, implying that stabilization of β-catenin is a major early event in colonic carcinogenesis. Similarly, in *Apc^{Min/+}* mice, the loss of the wild-type allele initiates adenoma formation (Moser *et al*, 1993; Oshima *et al*, 1995), and expression of an oncogenic form of β-catenin produces adenomas in the mouse intestine (Romagnolo *et al*, 1999).

Identification of the transcriptional targets of the Wnt/β-catenin signaling pathway is a potentially important means to understand the role of the canonical pathway in oncogenesis and development. A number of candidate target genes have been identified in human cell lines and tumors. (For more information on Wnt pathway targets, consult the Wnt webpage at www.stanford.edu/~rnusse/wntwindow.html.)

Our objective in the study reported here has been to identify novel β-catenin target genes that are regulated directly by DNA binding of β-catenin/TCF heterodimers and are potentially relevant to carcinogenesis. To this end, we employed microarray technology to identify genes with significantly altered levels of expression in human epithelial (293) cells expressing mutant (stabilized) β-catenin in which

serine 37 has been replaced with alanine; we then compared a list of these genes to a similar list obtained using the same methods to measure the abundance of RNAs in a well-characterized set of primary human ovarian endometrioid adenocarcinomas (OEAs) with and without Wnt pathway defects (Wu *et al*, 2001). Merging of microarray data from cell culture and OEA tumors revealed at least 17 genes that are regulated in common when similar criteria were applied. Two such genes, *FGF20*, a putative proto-oncogene (Jeffers *et al*, 2001), and *DKK1*, a Wnt pathway antagonist (Glinka *et al*, 1998), were studied in greater detail in mouse and human tumors, in frog development, in tests of the direct action of β-catenin-TCF heterodimers on gene expression, and in tests for a role of *FGF20* during maintenance of the β-catenin-induced transformed state. We present evidence supporting the conclusions that *FGF20* and *DKK1* are directly regulated by β-catenin during development and tumorigenesis, and that continued expression of *FGF20* is required to maintain the anchorage-independent growth state established by Wnt/β-catenin signaling.

Results

Genes regulated by mutant β-catenin in a human epithelial cell line

In an initial effort to identify genes regulated by mutant β-catenin, whether directly or indirectly, we used an efficient virus-based gene delivery system to introduce mutant β-catenin into virtually all cells in a cultured epithelial cell line, the human embryonic kidney cell line 293. This approach obviated a need to select individual clones of β-catenin-expressing cells and thus minimized variations in gene expression that might have been attributed to clonal variation.

293 cells were engineered to produce Tva, the avian leukosis subgroup A virus receptor, allowing efficient infection by the avian retroviral vector RCAS (Fisher *et al*, 1999). In addition, the β-catenin/TCF reporter construct, pOT, was inserted in the genome of this cell line to monitor β-catenin/TCF activity (Rubinfeld *et al*, 1993). The resulting cell line, 293Top, was infected with RCAS vectors encoding either GFP, to serve as a control, or HA-tagged β-cateninS37A, a stable mutant protein (Wu *et al*, 2001). Typically, about 80% of 293 cells were infected with either virus, as judged by anti-HA immunofluorescence or GFP fluorescence (data not shown). In the cells infected with RCAS-β-cateninS37A (293Top-S37A), the pOT reporter was typically induced 100–300-fold compared to cells infected with RCAS-GFP (293Top-GFP; data not shown).

RNA for microarray analysis was isolated 7 days after viral infection from four independently infected cultures, and chromophore-tagged cDNAs were hybridized to human Affymetrix U133A oligonucleotide microarrays to compare the messenger RNA (mRNA) expression profile of 293Top-S37A cells with that of 293Top-GFP cells, as described in greater detail in Materials and Methods. The criteria for identifying genes as up- or downregulated by β-cateninS37A included differential expression of at least two-fold, with a *P*-value of less than 0.05 using a parametric test.

In all, 62 genes were represented by higher levels of RNA in cells expressing β-cateninS37A than in GFP-expressing cells, and 15 genes were represented by lower RNA levels.

Genes previously reported to be regulated by β-catenin-mediated Wnt signaling, such as *CCND1* (CyclinD1), *ENC1*, *ABCB1* (MDR1), *LEF1*, *ENPP2* (Autotaxin), *MSX1*, and *MSX2*, were among the genes upregulated in the 293 cells expressing β-cateninS37A, as compared to the 293 cells expressing GFP (Supplementary Table 1S). Among the top 20 upregulated genes, we identified several known or suspected proto-oncogenes, such as *FGF20* (Jeffers *et al*, 2001), *ETV5* (Ets-5) (Dhulipal, 1997), *LMO2* (Rabbitts *et al*, 1997; Hacein-Bey-Abina *et al*, 2003), and some genes implicated in Wnt signaling, such as *DKK1* (Glinka *et al*, 1998) and *WNT11* (Table I). Reverse transcription followed by semiquantitative polymerase chain reaction-mediated amplification (RT–PCR assay) was used to confirm the findings, with some of the most dramatically upregulated genes by microarray tests (Figure 1A).

FGF20 mRNA is undetectable in 293Top cells that express GFP, although readily observed in cells expressing activated β-catenin. In a survey of normal human tissues with RT–PCR assays, *FGF20* RNA was found exclusively in the adult central nervous system, suggesting that expression of *FGF20* is tightly controlled in normal development. The gene is, however, expressed in human cancers; for example, *FGF20* RNA was detected in five of 15 human colon cancer cell lines (Jeffers *et al*, 2001). One of those lines, the SW480 line, is known to have deregulated β-catenin due to loss of *APC* (Munemitsu *et al*, 1995). We have corroborated this finding with SW480 cells, and we have also found *FGF20* RNA in the ovarian endometrioid cell line TOV112D, which contains mutant form of β-catenin (Figure 1B). In contrast, *FGF20* RNA is not detectable in TOV21G or LS123, ovarian and colorectal carcinoma lines respectively, harboring wild-type β-catenin (Rutzky *et al*, 1983; Wu *et al*, 2001; MN Chamorro, unpublished data, 2004; Figure 1B).

Prior studies have shown that activated mutants of β-catenin promote neoplastic transformation of RK3E cells, a rat epithelial cell line (Kolligs *et al*, 1999). In agreement with our expression profile of *FGF20* in 293Top cells, *FGF20* RNA is readily detectable in the β-catenin-transformed RK3E lines by RT–PCR, but not in the parental line (Figure 1C).

Increases in FGF-20 and DKK1 RNA are associated with deregulated β-catenin in human ovarian endometrioid adenocarcinomas

We next extended our findings with 293Top cells by examining the gene expression profiles of our large collection of OEAs. Approximately 40% of these tumors have mutations in *CTNNB1* (β-catenin), *APC*, *AXIN1* or *AXIN2* (Wu *et al*, 2001), providing a test of the proposed correlation between Wnt/β-catenin pathway defects and induction of candidate target genes for regulation by β-catenin, as illustrated previously for the β-catenin-regulated genes, *ITF-2* and *AXIN2* (Kolligs *et al*, 2002; Leung *et al*, 2002).

Affymetrix U133A oligo microarrays were again used to profile gene expression in 18 OEAs with an intact β-catenin pathway and 12 OEAs with deregulated β-catenin, in a fashion similar to recently published work using lower density HuGeneFL arrays (Schwartz *et al*, 2003). By comparing the profiles from tumors with normal versus mutant Wnt/β-catenin pathways, we identified a list of 563 genes differentially regulated by at least 1.75-fold with a *P*-value of less than 0.05 (Schwartz *et al*, 2003). To develop a shorter list of

Table I List of the twenty most strongly upregulated genes in 293Top cells by mutant β-cateninS37A

Gene symbol	Gene name	Fold change	*P*-value	Function
FGF20	Fibroblast growth factor 20	17.7	0.003	Signal transduction; cell–cell signaling
DKK1	Dickkopf (*Xenopus laevis*) homolog 1	15.5	<0.001	Extracellular Wnt signaling antagonist
MEGT1	Megakaryocyte-enhanced gene transcript 1	9.4	0.012	Unknown
BIK	BCL2-interacting killer	9.2	0.002	Apoptotic program; induction of apoptosis
EST	GenBank acc. # AK022120	9.1	0.014	Unknown
ETV5	Ets variant gene 5	6.2	0.007	Transcription factor
WNT11	Wingless-type MMTV integration site, member 11	5.3	0.006	Signal transduction; cell–cell signaling; embryogenesis; and morphogenesis
SLC2A3	Solute carrier family 2, member 3	5.2	0.026	Glucose transport; carbohydrate metabolism
SERPIND1	Serine proteinase inhibitor, clade D, member 1	5	0.049	Plasma glycoprotein; proteinase inhibitor
SLC7A8	Solute carrier family 7, member 8	4.9	0.007	Cationic amino-acid transporter
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	4.7	0.001	Cell motility; G-protein-linked receptor signaling pathway; transcription factor binding
DUSP6	Dual-specificity phosphatase 6	4.6	0.024	Apoptosis; MAPKKK cascade; cell cycle control; inactivation of MAPK
LMO2	LIM domain only 2 (rhombotin-like 1)	4.2	<0.001	Oncogenesis; developmental processes
SLC2A14	Solute carrier family 2, member 14	4.0	0.001	Carbohydrate transport
ARHGAP26	GTPase regulator associated with FAK	3.8	0.028	Neurogenesis; cell growth/maintenance
GAD1	Glutamate decarboxylase 1 (brain, 67 kDa)	3.6	0.003	Synaptic transmission; glutamate decarboxylation
FUT1	Fucosyltransferase 1	3.5	0.036	Carbohydrate metabolism
QPTC	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	3.5	<0.001	Protein modification
RBP1	Cellular retinol-binding protein 1	3.3	0.024	Retinoid binding; vitamin A metabolism
ABCB1	MDR/TAP member 1	3.1	0.002	Drug resistance; small-molecule transport

RNA for microarray analysis was isolated from 293Top cells 7 days after viral infection from four cultures independently infected with either RCAS-β-*cateninS37A* or RCAS-*GFP*. The top 20 upregulated genes are shown in descending order with respect to fold increase in RNA levels. *P*-values were obtained using a parametric test. Known or suspected proto-oncogenes and genes implicated in the Wnt pathway are shown in bold-faced letters.

genes that are commonly regulated by Wnt/β-catenin signaling in different cell types, we compared our list of regulated genes using modestly different criteria from 293Top cells (two-fold regulation, $P<0.05$) and OEAs (1.75-fold regulation, $P<0.05$). By these criteria, at least 17 genes appeared to be regulated in common; 16 of these genes were upregulated and one was downregulated (Table II). It is noteworthy that eight of the 20 most dramatically upregulated genes in 293Top cells are present in the combined list of 16 upregulated genes, including three proto-oncogenes and two genes involved in Wnt signaling (Tables I and II).

The two genes that appear to be most highly induced by stabilized β-catenin in both the 293Top cell system and in OEA primary tumors are *FGF20* and *DKK1* (Table II). Figure 2A demonstrates that both of these genes are expressed at markedly elevated levels in eight of 12 OEAs harboring mutations that deregulate β-catenin, but neither was induced in the 19 tumors lacking such mutations.

FGF-20 is expressed in adenomas from Apc^{Min/+} mice but not in normal intestinal mucosa

Of the suspected proto-oncogenes on our combined list of candidate targets for regulation by β-catenin, *FGF20* displayed the strongest correlation between RNA levels and the status of the Wnt/β-catenin pathway in OEA tumors. We therefore extended our studies of *FGF20* expression to other tumor types.

In mice heterozygous for loss-of-function mutations at the *Apc* locus, such as $Apc^{Min/+}$ mice and other heterozygous *Apc* knockout mice, loss of the wild-type allele initiates adenoma formation in the small intestine (Oshima *et al*, 1995). Using RT–PCR to detect expression of RNA, we found little or no *Fgf20* RNA in non-neoplastic intestinal

tissues from 15-day-old and adult $Apc^{Min/+}$ mice, but readily detected *Fgf20* RNA in all the adenomas analyzed (Figure 2B).

Stabilization of β-catenin in colonic epithelial cells by loss of *APC* is an early event in the majority of human colon cancers (Polakis, 2000). In preliminary experiments, we used the RT–PCR assay to measure *FGF20* RNA in six primary human colon adenocarcinomas and in three samples of normal colon mucosa. We found *FGF20* RNA in half of the tumors, but in none of the normal mucosas (Supplementary Figure IS).

XFGF20 and Xdkk-1 are downstream of the Wnt pathway in Xenopus laevis embryos

Much of our knowledge about the Wnt pathway and its role in vertebrate development comes from studies in *Xenopus laevis* (reviewed in Harland and Gerhart, 1997; Huelsken and Birchmeier, 2001). We therefore sought to extend the evidence for regulation of some of our candidate genes by the Wnt/β-catenin pathway by testing for transcripts in *Xenopus* embryos at relevant times in development. Both *FGF20* and *DKK1* were first identified in *X. laevis* (Glinka *et al*, 1998; Koga *et al*, 1999) and are closely related to their human counterparts (79% amino-acid identity between *Xenopus* and human FGF-20; 56% identity between Xdkk-1 and human DKK-1). *Xdkk-1* is expressed at the beginning of gastrulation (stage 10) in the future anterior endomesoderm, on the dorsal side of the embryo (Glinka *et al*, 1998). The localization of *XFGF20* RNA was previously reported for the neurula stage, but the RNA could be detected from late blastula onwards by RT–PCR (Koga *et al*, 1999). As in *Xenopus* early developmental stages are the most accessible experimentally, we confirmed zygotic expression from stage 10 onwards (Figure 3B, left panel), and localized *XFGF20* RNA by RT–PCR exclusively to the equatorial region (the marginal
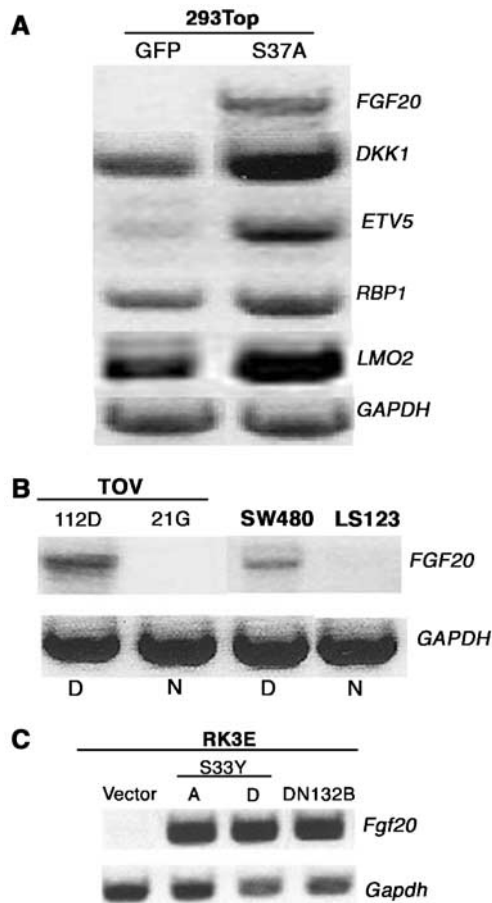
Figure 1 Validation of microarray data by semiquantitative RT–PCR. (**A**) To verify oligonucleotide microarray results, semi-quantitative RT–PCR was used to estimate the amount of RNA from five of the upregulated genes in 293Top cells (Table I). The same total RNA sample was used to prepare probes for microarray hybridization. (**B**) Expression of *FGF20* RNA as assessed by RT–PCR with RNA from OEA cell line TOV112D, ovarian clear cell carcinoma-derived line TOV21G, and colon cancer cell lines SW480 and LS123. The letters D and N refer to β-catenin status (Deregulated and Normal). (**C**) Measurement of *FGF20* RNA from RK3E cells transformed by β-cateninS33Y (clones A and D) and N-terminal-deleted β-cateninΔN132 (ΔN132B) by RT–PCR. *GAPDH* mRNA was reverse transcribed and amplified to control for the amount of RNA loaded.

zone) of stage 10 embryos, which is fated to produce meso-derm, (Figure 3B, right panel). The pattern of *XFGF20* expression is similar to that described for other *FGF* genes like *eFGF* and *XFGF3* (Isaacs *et al*, 1994; Schohl and Fagotto, 2003), and for a group of genes expressed in the marginal zone, which require a zygotic Wnt pathway (Figure 3A), such as *XmyoD* and *Xbra* (Hoppler *et al*, 1996; Vonica and Gumbiner, 2002).

Activating or inhibiting the Wnt pathway had a dramatic effect on *FGF20* and *DKK1* expression. We injected RNA encoding an activated form of the downstream component of the pathway (VP16Xtcf-3) or a dominant-negative mutant (DNXtcf-3) (Vonica *et al*, 2000) into the equatorial zone of each blastomere at the four-cell stage (Figure 3C). *XFGF20* and *Xdkk-1* RNAs were increased by *VP16Xtcf-3*, and unde-tectable when Wnt signaling was blocked with *DNXtcf-3*RNA. Known direct (*siamois*) (Carnac *et al*, 1996; Brannon *et al*,

1997) and indirect (*chordin*) (Kessler, 1997) target genes of the maternal dorsal Wnt pathway (Figure 3A), as well as the zygotic Wnt target and mesodermal marker *Xbra* (Smith *et al*, 1991; Vonica and Gumbiner, 2002), showed a similar re-sponse. In contrast, *Xwnt-8*, a marker of ventral mesoderm (Christian *et al*, 1991) inhibited by the maternal Wnt path-way, retained expression in embryos injected with *DNXtcf-3* RNA, but was absent when *VP16Xtcf-3* RNA was injected.

We also asked whether ectopic activation of the Wnt pathway in animal cap explants, which are normally fated to produce only ectoderm, could induce expression of *XFGF20* and *Xdkk-1* (Figure 3D). On its own, VP16Xtcf-3 can induce both genes only slightly, but induction was significantly enhanced by co-injection of the endomesoderm inducer *VegT* (Kofron *et al*, 1999). The indirect target *chordin* was similarly activated, and the direct Wnt target *twin*, a homo-logue of *siamois*, was strongly activated with *VP16Xtcf-3* alone. We conclude that expression of *XFGF20* and *Xdkk-1* in animal caps is augmented by Wnt signaling. The differ-ences in embryonic expression patterns between the two genes could be a consequence of different requirements for cooperative signaling pathways, responses to different Wnt ligands, or both.

FGF-20 and DKK1 are direct targets of the canonical Wnt signaling pathway

To differentiate between the direct and indirect effects of β-catenin stabilization on the control of *FGF20* and *DKK1*, we took advantage of a dexamethasone-inducible form of VP16 Xtcf-3 (TVGR) (Darken and Wilson, 2001). *TVGR* RNA was injected alone or in combination with *VegT* RNA in the animal pole of two cell stage *Xenopus* embryos (Figure 4). Animal caps were dissected at stage 8.5 and dexamethasone was added in the presence or absence of cycloheximide, an inhibitor of translation. Under these conditions, the transcrip-tion of only those genes directly activated by VP16 Xtcf-3 will increase upon addition of the inducer. Cycloheximide treatment decreased the levels of *XFGF20* and *Xdkk-1* RNA in *VegT* and *TVGR*-injected embryos, but addition of dexa-methasone significantly raised them. *siamois*, a direct Tcf target, and, less dramatically, *Xbra*, showed similar variation. On the contrary, levels of *chordin* RNA, an indirect Wnt target, did not respond to dexamethasone when translation was inhibited. The low level of *siamois* induction and the absence of *chordin* stimulation upon addition of dexametha-sone are due to the late timing of Wnt activation, at the limit of competence for activation of dorsal genes (Darken and Wilson, 2001). In conclusion, the injection experiments in frog embryos suggest that expression of *XFGF20* and *Xdkk-1* is subject to direct regulation by the Wnt/β-catenin signaling pathway.

To address the implied direct relationship between the Wnt/β-catenin pathway and the regulation of *FGF20* and *DKK1* more rigorously, we have performed chromatin immu-noprecipitation (ChIP) assays with an anti-β-catenin antibody in the 293Top cells (for ChIP methodology, see Orlando, 2000; Weinmann *et al*, 2001). Stabilized β-catenin accumulates in the nucleus in a complex with TCF, and the heterodimer influences transcription by binding to TCF recognition sites in DNA (Huelsken and Behrens, 2002). Regulatory domains of direct β-catenin/TCF targets are therefore expected to be enriched in anti-β-catenin immunoprecipitates, as compared

Table II List of genes regulated by β-catenin in both 293Top cells and in OEAs

Gene symbol	Gene name	293Top fold change	*P*-values	OEAs fold change	*P*-values
FGF-20	Fibroblast growth factor 20	17.70	0.003	7.39	<0.001
DKK1	Dickkopf homolog 1 (*Xenopus laevis*)	15.55	<0.001	12.95	<0.001
ETV5	Ets variant gene 5 (ets-related molecule)	6.23	0.007	1.82	0.037
WNT11	Wingless-type MMTV integration site family, member 11	5.32	0.006	2.20	0.011
LMO2	LIM domain only 2 (rhombotin-like 1)	4.27	<0.001	4.05	<0.001
GAD1	Glutamate decarboxylase 1	3.66	0.003	6.89	<0.001
QPCT	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	3.57	<0.001	3.90	<0.001
ABCB1	ATP-binding cassette, subfamily B (MDR/TAP), member 1	3.15	0.002	1.75	<0.001
SNK	Serum-inducible kinase	2.75	0.006	3.49	<0.001
IRS1	Insulin receptor substrate 1	2.62	0.002	2.68	0.001
TBX3	T-box 3 (ulnar mammary syndrome)	2.49	<0.001	2.17	0.024
MSX2	Msh homeo box homolog 2 (*Drosophila*)	2.47	<0.001	6.03	0.000
MSX1	Msh homeo box homolog 1 (*Drosophila*)	2.46	0.001	3.31	0.047
DNCI1	Dynein, intermediate polypeptide 1	2.20	<0.001	3.70	<0.001
CCND1	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	2.05	<0.001	2.90	0.001
NMA	Putative transmembrane protein	2.01	0.003	7.28	<0.001
ISYNA1	Myo-inositol 1-phosphate synthase A1	−2.1	<0.001	−2.1	0.009

Regulated genes are shown in descending order with respect to fold change in upregulation in 293Top cells.
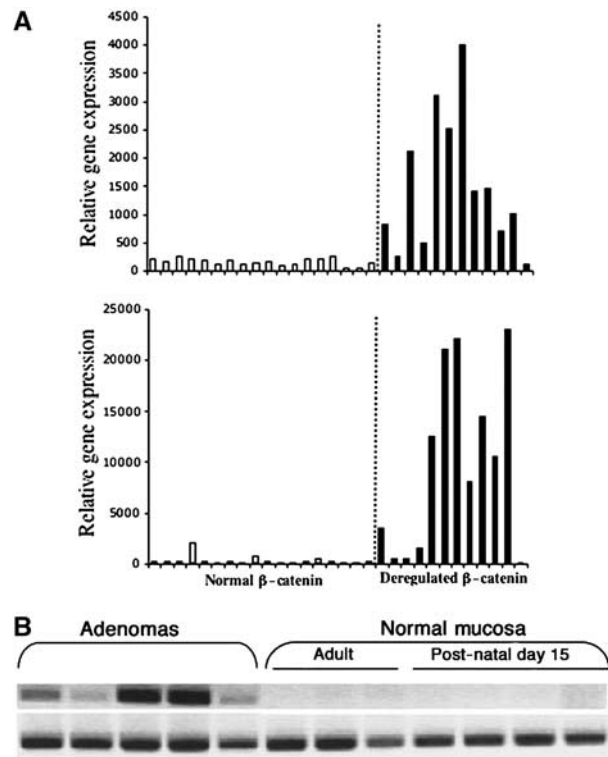

Figure 2 *FGF20* and *DKK1* gene expression in individual human and mouse tumors. (**A**) *FGF20* (upper panel) and *DKK1* (lower panel) gene expression in OEAs. Relative RNA levels were determined by Affymetrix microarray data analysis. White boxes represent the relative gene expression in tumors with an intact Wnt pathway and black boxes represent tumors with a deregulated Wnt pathway. (**B**) *FGF20* RT–PCR products using 200 ng of RNA from adenomas from *Apc*^*Min/+*^ mice and from normal intestinal mucosa samples from 15-day-old and adult *Apc*^*Min/+*^ mice.

Figure 3 Wnt signaling regulates *XFGF20* and *Xdkk-1* expression in *Xenopus* embryos. (**A**) Schematic description of a pregastrula *Xenopus* embryo (left panel) and the localizations and timing of the maternal and zygotic Wnt pathways (right panel). (**B**) *XFGF20* is expressed zygotically and localized exclusively in the marginal zone. RT–PCR for *XFGF20* in various developmental stages (left panel) and various locations of stage 10 (early gastrula) embryos (right panel). *ODC* RNA and *Xwnt-8* RNA serve as controls for amounts loaded and localization (*ODC* is normally expressed weakly in vegetal cells). (**C**) Modulation of the Wnt signaling pathway alters *XFGF20* and *Xdkk-1* expression. Embryos were injected marginally in each cell at the four-cell stage with 100 pg *DN-Xtcf-3* RNA or 20 pg *VP16-Xtcf-3* RNA, and collected at stage 9.5 for RT–PCR analysis. *XFGF20*, *Xdkk-1*, *siamois*, *chordin*, and *Xbra* all required early activation of the Wnt pathway, while *Xwnt-8* is repressed under the same conditions. (**D**) Synergistic effect of the Wnt pathway and the endomesoderm inducer *VegT* on ectopic expression of *XFGF20* and *Xdkk-1* in animal caps. Two-cell stage embryos were injected in the animal pole of each cell with 400 pg *VegT* RNA or 10 pg *VP16 Xtcf-3* RNA, as indicated, and animal caps were cut and collected at stage 9.5 for RT–PCR analysis.

to immunoprecipitates obtained with a control antibody. Formaldehyde-fixed chromatin from 293 cells expressing either β-cateninS37A or GFP was subjected to immunoprecipitation with either a rabbit anti-β-catenin antibody or an

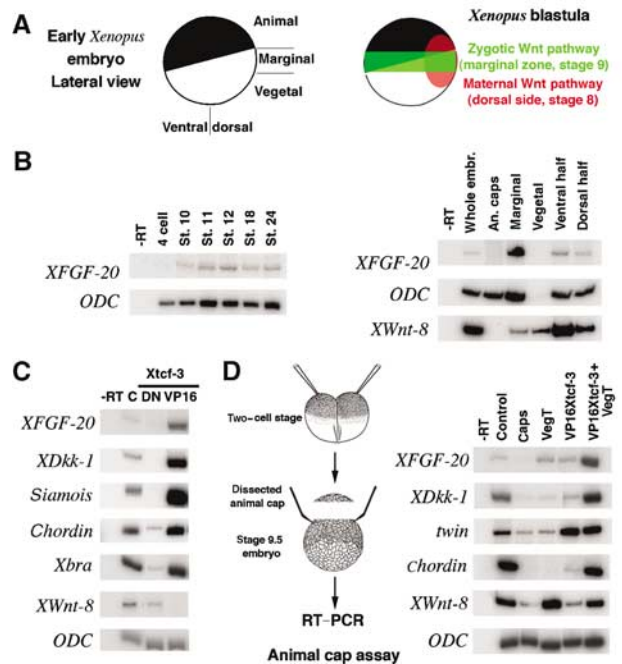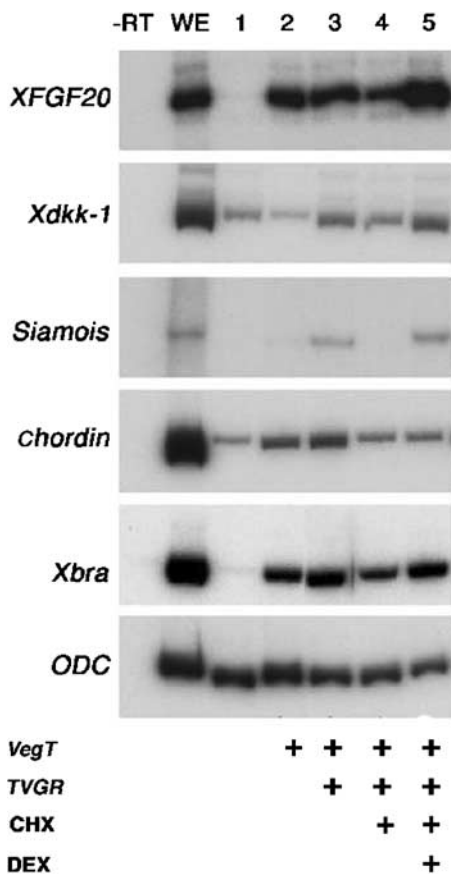**Figure 4** *Xenopus XFGF20* and *Xdkk-1* are direct targets of the Wnt pathway. Embryos were injected at the two-cell stage in both blastomeres with 400 pg *VegT* RNA and 20 pg TVGR RNA, and treated with cycloheximide (CHX) and dexamethasone (DEX) as indicated. XFGF20 and *Xdkk-1* were induced when DEX was added to CHX-treated caps (compare lanes 4 and 5). *Siamois* is a control for direct induction by the Wnt pathway, and *chordin* for indirect induction.

irrelevant rabbit antibody as a negative control. To determine whether immunoprecipitates were enriched for regulatory DNA sequences from the putative target genes *DKK1* and *FGF20*, we used PCR to amplify the precipitated DNA and determined the amounts of *DKK1* and *FGF20* regulatory regions as well as DNA from the presumably irrelevant genes *GAPDH* and *LMO7*, neither of which shows evidence of regulation by Wnt/β-catenin signaling. As illustrated in Figure 5, the approximately 500 bp regions immediately upstream of the transcript start sites of the *DKK1* and *FGF20* genes (see Figure 6A for amplified promoter regions) were enriched in the anti-β-catenin immunoprecipitates from cells expressing S37A β-catenin, compared to immunoprecipitates formed with control antibody; no differences were seen with tests of the promoter regions of *GAPDH* and *LMO7*. Moreover, no differences in the amounts of these putative regulatory domains were seen with cells expressing GFP. In addition, as positive controls, we showed that β-catenin–DNA complexes from 293Top cells expressing β-cateninS37A are enriched for the promoter regions of the pOT reporter and from the *CCND1* (Cyclin D1) gene (Figure 5); the latter has been shown to be a direct target of the β-catenin/TCF complex (Tetsu and McCormick, 1999). Our ChIP data strongly suggest that
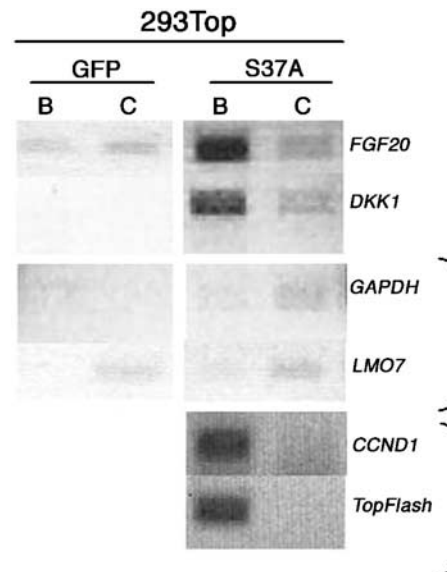

Figure 5 ChIP assay in 293Top cells shows that *FGF20* and *DKK1* are direct targets of β-catenin. Formaldehyde-fixed chromatin from 293Top cells infected either with RCAS-β-*catenin*S37A or RCAS-*GFP* was immunoprecipitated either with anti-β-catenin antibody (**B**) or with control isogenic antibody (**C**). The immunoprecipitated DNAs were PCR-amplified using primers mapping to the proximal promoter sequences of *FGF20* and *DKK1*. Amplification of the *GAPDH* and *LMO7* promoters was used as a negative control, and amplification of the TOPFLASH and *CCND1* (Cyclin D1) promoters was used as a positive control.

DKK1 and *FGF20*, as well as *CCND1* and the pOT reporter, are direct targets of β-catenin.

β-Catenin regulates FGF-20 and DKK1 transcription in 293 cells and Xenopus embryos via TCF-binding sites proximal to the promoter

To further investigate the regulation of *DKK1* and *FGF20* by β-catenin, genomic fragments containing regions 5′ to the transcriptional start sites were placed upstream of a *luciferase* reporter gene (*DKK1-luciferase* and *FGF20-luciferase*); these constructs were then used to study regulation of luciferase production by mutant β-catenin in 293 cells and in *Xenopus* embryos.

Inspection of the sequences of the putative regulatory regions revealed four potential TCF-binding sites (CTTTGA/TA/T) in *DKK1* and two in *FGF20* (Figure 6A). Transient co-transfection of 293 cells with either of the *luciferase* reporters and a plasmid encoding β-cateninS37A stimulated the *DKK1-luciferase* reporter about five-fold and the *FGF20-luciferase* reporter about two-fold. These effects were abolished when ICAT, a β-catenin inhibitor (Tago *et al*, 2000), or the C-terminus of E-cadherin, which binds to β-catenin, was cotransfected with β-cateninS37A (Figure 6A). The low activity of the *FGF20-luciferase* reporter suggests that it probably lacks other regulatory sequences for optimal response to mutant β-catenin. Parallel tests with a semi-quantitative RT–PCR assay showed that mutant β-catenin increased the levels of *DKK1* RNA from the endogenous gene to the same extent as it increased the luciferase activity from the *DKK1* reporter construct (data not shown), suggesting that the *DKK1* reporter contains most or all of the sequences necessary for regulation by β-catenin.
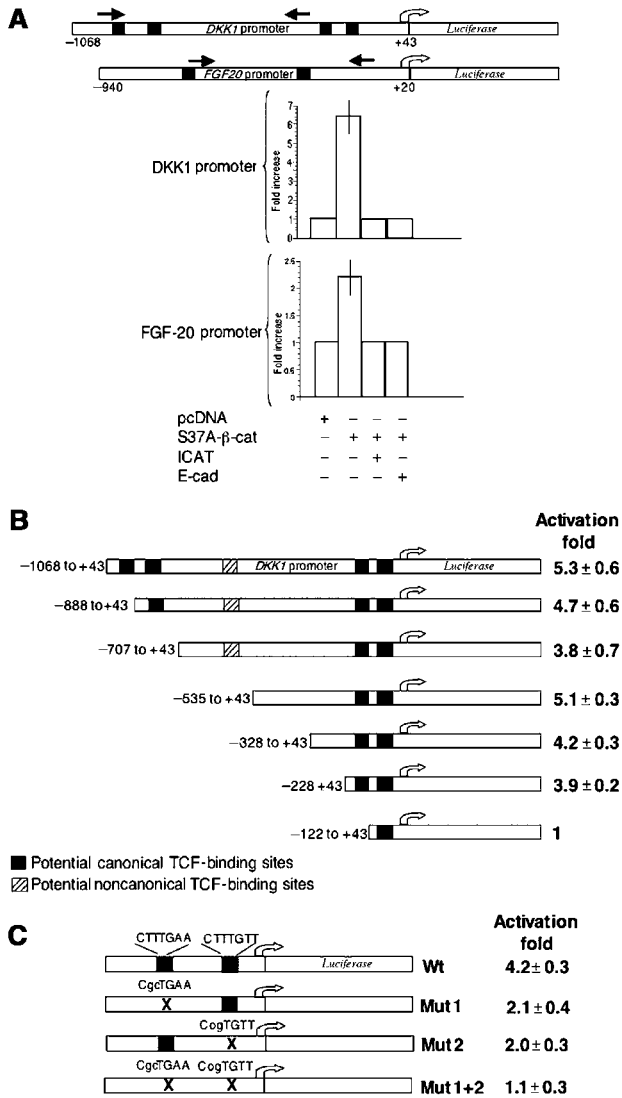
A

pcDNA   +   −   −   −
S37A-β-cat   −   +   +   +
ICAT   −   −   +   −
E-cad   −   −   −   +

B

Construct	Activation fold
−1068 to +43	5.3 ± 0.6
−888 to +43	4.7 ± 0.6
−707 to +43	3.8 ± 0.7
−535 to +43	5.1 ± 0.3
−328 to +43	4.2 ± 0.3
−228 +43	3.9 ± 0.2
−122 to +43	1

■ Potential canonical TCF-binding sites
▨ Potential noncanonical TCF-binding sites

C

Construct		Activation fold
CTTTGAA CTTTGTT	Wt	4.2 ± 0.3
CgcTGAA	Mut 1	2.1 ± 0.4
CGgTGTT	Mut 2	2.0 ± 0.3
CgcTGAA CGgTGTT	Mut 1+2	1.1 ± 0.3

Figure 6 Mapping regions required for response to β-catenin in human *DKK1* and *FGF20* promoters reporter constructs in 293 cells. (**A**) Depicted at the top are schematic diagrams of the promoter reporter constructs; black boxes represent potential LEF/TCF binding sites. Black arrows represent location of primers used to amplify immunoprecipitated chromatin in the ChIP assays. The relative activity of the promoter constructs was assessed in 293 cells after transient co-transfections with either empty vector or vector expressing β-cateninS37A in the presence or absence of vectors expressing either ICAT or the C terminus of E-cadherin. To control for transfection efficiency, firefly luciferase activity was normalized to results with *Renilla* luciferase. (**B**) *DKK1* promoter deletion analysis. 5′ deletions of pDKK1-*luciferase* were generated using PCR, and their relative activities were measured after transient co-transfections with either empty vector or vector expressing β-*catenin*S37A, as described in Materials and methods. (**C**) Mutagenesis analysis of potential LEF/TCF-binding sites in the −−328 to +43 DKK1-luciferase construct. Mutations in the putative TCF-binding sites were generated using QuickChange Site Directed Mutagenesis Kit (Stratagene), and their relative activities assessed as described for panel B.

A series of 5′ deletions was then used to map the regions of the regulatory domain of the *DKK1* promoter required for β-catenin stimulation. No significant changes were observed until the deletions reached the region containing the two TCF-binding sites proximal to the start site (Figure 6B). Site-

directed mutations of either of these two sites partially impaired the response β-catenin, while mutations affecting both sites resulted in complete loss of activity (Figure 6C).

Sequence conservation in the regulatory elements of vertebrate promoters is well documented to have occurred during evolution. We therefore tested *luciferase* reporter constructs containing the human *FGF20* and *DKK1* promoters in transcription assays in *Xenopus* embryos (Figure 7). Both wild-type reporters responded strongly to co-injected *VP16 Xtcf-3* RNA (Figure 7A and B), while the TCF site-mutated reporters were considerably less sensitive, demonstrating that the Wnt effect is mediated mainly by these sites. In addition, the human *FGF20* promoter showed high levels of activity when injected alone in *Xenopus* embryos, with the greatest activity in the marginal zone, corresponding to the normal pattern of expression of the *Xenopus* homologue (not shown). This activity was also dependent on intact TCF sites, as the reporter with the mutant TCF sites was significantly less active (Figure 7C). We note that at stage 9 the wild-type *FGF20* promoter was more active dorsally, probably due to an earlier onset of transcription there, as has been reported for *Xbra* (Lerchner *et al*, 2000; Vonica and Gumbiner, 2002).

FGF-20 small inhibitory RNA (siRNA) interferes with β-catenin-mediated transformation of RK3E cells

Mutant β-catenin promotes the neoplastic transformation of RK3E cells and renders them capable of anchorage-independent growth, as judged by formation of colonies in soft agar (Kolligs *et al*, 1999). As shown above, RK3E cells transformed by β-catenin express *Fgf20* (Figure 1), and FGF-20 has been reported to induce morphological transformation of mouse 3T3 cells (Jeffers *et al*, 2001). To evaluate the possibility that production of FGF-20 is required to maintain the transformed phenotype of RK3E cells expressing ectopic β-catenin, we attempted to block the expression of the *FGF20* gene in those cells using siRNAs (Brummelkamp *et al*, 2002).

To this end, we constructed plasmids encoding two siRNA hairpins based on the rat *FGF20* gene sequence (siFGF20.A and siFGF20.B); an additional plasmid (siFGF20.C) encodes a mutant form of one of these siRNAs. We initially showed that siFGF20.A and siFGF20.B could reduce the amounts of FGF-20 protein in RK3E cells programmed to express a V5-tagged version of rat FGF-20 (RK3E-FGF20V5; Figure 8A). Subsequently, the two wild-type constructs and the mutant construct, siFGF20.C, were transfected into RK3E cells stably transformed by DNA encoding a mutant β-catenin (clone S33YA; Figure 1). Selection for a co-transfected drug resistance gene generated three polyclonal cultures, RK3E-S33YA-siFGF20.A, RK3E-S33YA-siFGF20.B, and RK3A-S33YA-siFGF20.C, which were used in the subsequent experiments. The cells producing either of the wild-type siRNAs for rat *FGF20* contained relatively low levels *of FGF20* RNA as compared to the line producing the mutant siRNA, RK3A-S33YA-siFGF20.C, and the starting line, S33YA (Figure 8A and data not shown). When we attempted to grow cells from each of these cultures in soft agar, we found that the cells in which FGF-20 production was inhibited (RK3E-S33YA-siFGF20.A and RK3E-S33YA-siFGF20.B) formed few colonies, whereas the parental cell line and cells expressing the mutant form of *FGF20* siRNA formed colonies efficiently in soft agar

(Figure 8C). We conclude that continued production of FGF-20 is necessary for maintenance of the anchorage-independent growth capacity of RK3E cells expressing mutant β-catenin.
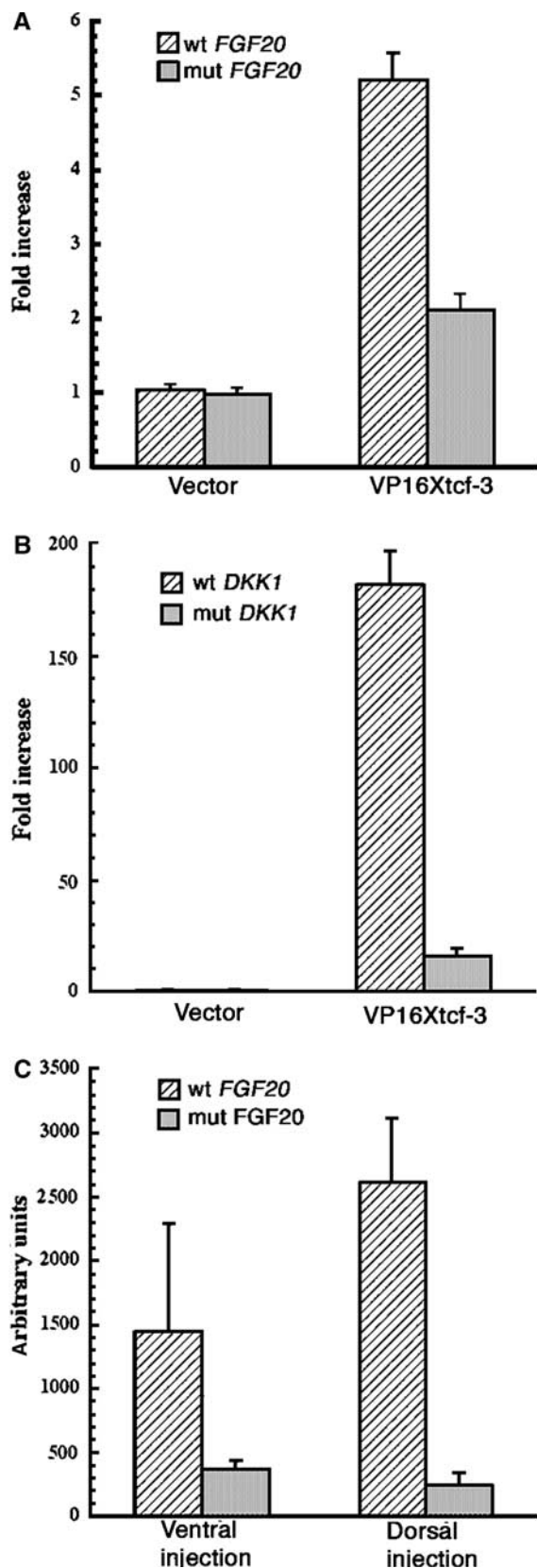
Discussion

The diverse phenotypic changes that occur in different cell types in response to signaling through the Wnt/β-catenin pathway are thought to result principally from altered regulation of transcription rates in response to changes in the nuclear concentration of β-catenin/TCF heterodimers. Consequently, many recent studies have been directed towards the identification of genes that are represented in mRNA pools at higher or lower levels when the Wnt/β-catenin pathway is activated (Conacci-Sorrell *et al*, 2002; Kielman *et al*, 2002; van de Wetering *et al.*, 2002; Willert *et al*, 2002; Schwartz *et al*, 2003; Shimokawa *et al*, 2003). Since this pathway governs crucial developmental events and contributes to neoplasia in a variety of cell lineages in many metazoan organisms, these studies of transcriptional control are likely to yield complex results, including the identification of genes that are secondarily regulated by Wnt/β-catenin signaling.

In this study, we have identified and focused on a small number of genes that appear to be strongly induced at the transcriptional level when β-catenin concentrations rise under several biological circumstances: ectopic expression of mutant β-catenin in cultured cell lines; mutations affecting components of the Wnt/β-catenin pathway in cancers arising in intestinal and ovarian cell types; and developmental events involving Wnt/β-catenin signaling in normal *Xenopus* embryos. Our study was designed to identify novel genes directly regulated by β-catenin that are potentially relevant to cancer development. To achieve this goal, we employed microarray technology to identify genes up- and downregulated, directly or indirectly, in human epithelial (293) cells expressing mutant (stabilized) β-catenin versus control GFP vector. We then compared this list of genes to a similar list obtained by assessing gene expression in a well-characterized set of primary human OEAs with and without Wnt pathway defects. ChIP assays in 293 cells and experiments in frog embryos using an inhibitor of translation were then undertaken to determine whether candidate genes were likely to be direct or indirect tagets of β-catenin/TCF.

We have chosen for special attention two genes that have not previously been reported to be regulated by Wnt/β-catenin signaling, *DKK1* and *FGF20*. These two genes are highly induced by stabilized β-catenin in both the 293Top cell system and in OEA primary tumors (Table II). Both of these genes are expressed at markedly elevated levels in eight of 12 OEAs tumors harboring mutations that deregulate β-catenin, but neither was induced in the 19 tumors lacking such

Figure 7 Activation of the human *FGF20* and *DKK1* promoters by VP16 Xtcf-3 in *Xenopus* embryos is dependent on intact TCF-binding sites. (**A**) VP16 Xtcf-3 activates the human *FGF20* promoter. Two-cell stage embryos were injected in the animal pole with 20 pg *FGF20-luciferase* reporter plasmids and 20 pg VP16 Xtcf-3 RNA, and collected at stage 10 for luciferase assays. Mutating the TCF-binding sites reduced activation from 5.2- to 2.1-fold. (**B**) Activation of the human *DKK1* promoter by VP16 Xtcf-3 in *Xenopus* requires intact TCF-binding sites. In all, 40 pg of wild-type of mutant mouse *DKK1-luciferase* reporter gene were co-injected with 100 pg VP16 Xtcf-RNA in the animal pole at the two-cell stage and recovered at stage 10. Mutating the TCF-binding sites reduces activation from 180- to 16-fold. (**C**) Early activation of the human *FGF20* promoter by *Xenopus* endogenous factors is dependent on functional TCF sites. Embryos injected dorsally or ventrally with 40 pg wild-type or mutant reporter DNA were recovered at stage 9 for luciferase assays.
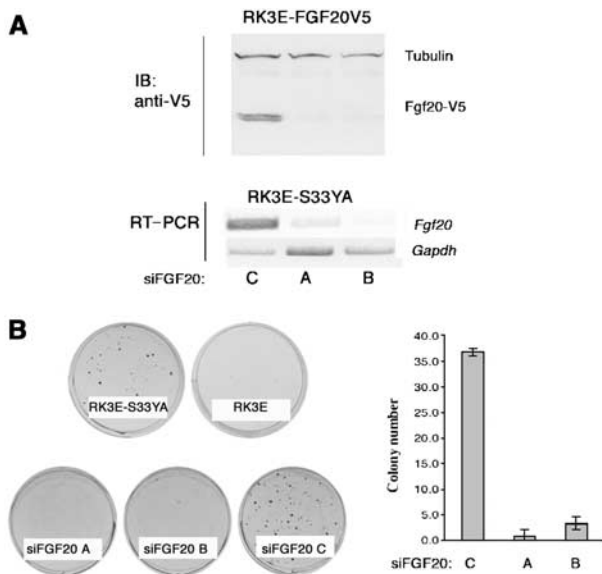
Figure 8 *FGF20* siRNA interferes with β-catenin-mediated growth of RK3E cells in soft agar. (**A**) Effect of *FGF20* siRNAs on RK3E cells. Top panel: RK3E cells expressing a v5-tagged version rat *FGF20* were transiently transfected with wild-type (lanes A and B) and mutant (lane C) rat *FGF20* siRNAs. Lower panel: semiquantitative RT–PCR was carried out with RNAs from RK3E-S33YA cells stably transfected with *FGF20* siRNAs. (**B**) Soft agar assay. 10^5 cells were plated in 0.4% agarose and incubated for 3 weeks. Cultures were fixed with 1% paraformaldehyde and stained with 0.1% methylene blue and counted. RK3E-S33YA cells form colonies in soft agar as opposed to RK3E cells. Stable RK3E-S33YA cells expressing wild-type *FGF20* siRNAs (siFGF20.A and siFGF20.B) formed colonies at a lower efficiency than cells expressing the scrambled *FGF20* siRNA. Bar graph at the left represents the number of visible colonies obtained for each cell line from a typical experiment performed in triplicates.

mutations. We do not know why some tumors with deregulated β-catenin do not have increased levels of *FGF20* or *DKK1* RNA. One possible explanation is that these genes are under the control of other pathways in addition to the Wnt/β-catenin pathway; therefore, it is the integration of signals in the nucleus that dictates the outcome in some cases. Indeed, it has been shown that *DKK1* is also regulated by Bmp signaling and the AP-1 family member c-jun (Grotewold and Ruther, 2002). Another explanation would be that the tumors without elevated *FGF20* and *DKK1* RNAs might have lower levels of β-catenin activity. Very little is known about the regulation of FGF-20, but our data from *Xenopus* experiments (see below) suggest that it is probably complex. However, in most tumors we studied, activation of the Wnt/β-catenin pathway correlated with augmented expression of these two genes.

We have shown, using ChIP assays, that *FGF20* and *DKK1* are direct targets of β-catenin in 293Top cells. We have also demonstrated the direct target relationship between the Wnt pathway and expression of *XFGF20* and *Xdkk-1* in *X. laevis* using a dexamethasone-inducible form of VP16-Xtcf3 in the presence of dexamethasone and cyclohexamide, and have defined the minimal region necessary for β-catenin regulation of the *DKK1* promoter in 293 cells using nested deletions. Mutational analysis of both promoters revealed that they are dependent on LEF/TCF-binding sites in 293Top cells and *Xenopus* embryos.

Our observations in frog embryos demonstrate that Wnt activation of *FGF20* and *DKK1* reflects a relation between the Wnt/β-catenin pathway and these targets during normal development; thus, upregulation of these is not an anomaly resulting from highly deregulated β-catenin activity in tumors and in cultured cells. However, it is unlikely that expression of these two genes in intact embryos at the late blastula–early gastrula stages depends on the same Wnt ligand, since patterns of expression for *Xdkk-1* and *XFGF20* in late blastula and early gastrula *Xenopus* embryos differ markedly. While *Xdkk-1* is present from stage 10 in anterior endomesoderm, forming the leading edge of the dorsally ingressed cells during gastrulation (Glinka *et al*, 1998), we find *XFGF20* to be expressed in the marginal zone, in a territory fated to become mesoderm after gastrulation. The pattern of *XFGF20* expression fits a pattern common to an increasing number of genes expressed in the marginal zone. These genes, which have been linked to a zygotic marginal Wnt pathway, include *XmyoD* (Hoppler *et al*, 1996; Hamilton *et al*, 2001), *Xpo* (Hamilton *et al*, 2001), *Xbra* (Vonica and Gumbiner, 2002), and *XFGF3* (Schohl and Fagotto, 2003).

The induction of *DKK1* by the Wnt/β-catenin pathway is of particular interest because *DKK1* encodes a secretory protein that serves as a negative regulator of Wnt binding to the LRP component of the receptor complex (Glinka *et al*, 1998; Semenov *et al*, 2001). This implies that signaling events initiated by production of Wnt proteins during normal development might be terminated by production of this inhibitor. In contrast, activation of the Wnt/β-catenin pathway during tumorigenesis by mutations affecting intracellular components of the pathway would be resistant to this auto-regulatory loop. Although upregulation of *DKK1* by mutated β-catenin in tumors should not affect β-catenin/TCF activity in cells harboring the oncogenic mutation, it might affect Wnt signaling to stromal cells, as recently suggested by the observation that myeloma cells producing DKK-1 are often associated with lytic bone lesions in patients with multiple myeloma (Tian *et al*, 2003).

DKK1 and other genes encoding extracellular inhibitors of Wnt signaling are not the only means for auto-inhibition of Wnt signaling. Wnt signaling is also subject to negative feedback regulation in the cytoplasm. For instance, Conductin/Axin2, which binds to β-catenin and induces its degradation, has been shown to be a direct transcriptional target of β-catenin/TCF (Jho *et al*, 2002; Leung *et al*, 2002). The ubiquitin receptor ligase, β-TrCP, which is involved in targeting β-catenin to proteosomes, is also activated by Wnt/β-catenin signaling (Spiegelman *et al*, 2000). In addition, Wingless, the Wnt-1 ortholog in *Drosophila*, induces the direct expression of *naked cuticle*, which inhibits Dishevelled and blocks further signaling (Rousset *et al*, 2001).

FGF20, the other gene induced by Wnt/β-catenin signaling that we have studied intensively in this report, belongs to a large class of genes, the *FGF*s, encoding secretory proteins with potent mitogenic and angiogenic roles in embryonic development, wound healing, and tumor development (reviewed in Dickson *et al*, 2000; Powers *et al*, 2000). Furthermore, activation of expression of *FGF3*, *FGF4*, and *FGF8* by the mouse mammary tumor virus (MMTV) initiates tumor development in the mammary glands of infected female mice, in a manner similar to activation of *Wnt* genes. The recently discovered *FGF20* gene is expressed

during early embryonic development and exclusively in the nervous system in adults (Koga *et al*, 1999; Jeffers *et al*, 2001). Ectopic overexpression of *FGF20*, as for other members of the *FGF* family, has been shown to transform cells in culture and make them capable of forming tumors in *nude* mice (Jeffers *et al*, 2001). Some *FGF*s in addition to *FGF20* also appear to be transcriptional targets for Wnt/β-catenin signaling. *FGF4* has recently been reported to be a direct target of β-catenin (Kratochwil *et al*, 2002), and *FGF9* RNA was reported to be present at elevated levels in OEAs with deregulated β-catenin (Schwartz *et al*, 2003). In *Xenopus*, expression of *XFGF3* was shown to be regulated by the maternal β-catenin activity (Schohl and Fagotto, 2003). Recently, *FGF18* was also shown to be a direct target of β-catenin and upregulated in human colon cancers; furthermore, interference with expression of *FGF18* suppresses the growth of colon cancer cells in culture (Shimokawa *et al*, 2003).

Based on the evidence of the involvement of FGFs in tumorigenesis, we have asked whether cell transformation by mutant β-catenin depends on induction of *FGF20*. Using plasmids encoding inhibitory hairpin RNAs directed against *FGF20* RNA, we have found that, when concentrations of *FGF20* RNA and protein are reduced in β-catenin transformed rat epithelial cells, the capacity to form anchorage-independent colony is also reduced (Figure 8). The potential relevance of activated *FGF20* in β-catenin-induced cancers is further supported by our finding that *FGF20* is actively expressed in all five adenomas examined from Min mice, and by our preliminary observations that a subset of human colon cancers and cell lines express *FGF20* RNA, whereas normal colon tissues do not (Supplementary data). This is consistent with a large body of evidence that deregulated β-catenin signaling is common in such tumors (Polakis, 2000).

FGF20 and members of the FGF family are not the only presumptive or known proto-oncogenes reported to be induced by Wnt/β-catenin signaling. For example, two well-established proto-oncogenes, *c-MYC* (He *et al*, 1998) and *CCND1* (CyclinD1) (Shtutman *et al*, 1999; Tetsu and McCormick, 1999) have been shown to be directly targeted by β-catenin/TCF complexes in colorectal tumor cell lines with *APC* mutations or activating β-*catenin* mutations. An oncogenic role for these proto-oncogenes in cancers in which β-catenin is deregulated is possible but not yet proven. On the other hand, genetic experiments with *CCND1*-deficient mice suggested that Cyclin D1 is not essential for intestinal tumorigenesis in animals carrying the *Apc^{min}* allele (Wilding *et al*, 2002). Analogous experiments with *FGF20* null mutants might help to establish the role this gene plays in Wnt/β-catenin-mediated tumorigenesis in the colon, breast and other organs. If *FGF20* is commonly induced in various human neoplasms, it might provide a useful marker for detecting cancer through noninvasive methods; in addition, if the gene has a functional role in tumorigenesis, it might also be a useful and accessible therapeutic target.

We concentrated on *FGF20* and *DKK1* because they were dramatically upregulated in two different settings—a cultured human cell line expressing mutant β-catenin and ovarian tumors with mutations in the Wnt signaling pathway—as observed when we merged lists of Wnt/β-catenin-regulated genes generated by studies being undertaken in our two laboratories. In addition, we chose to study these genes

because *FGF20* and *DKK1* have not been previously reported to be directly regulated by this signaling pathway, and because they are likely to be important targets for transcriptional control in view of their putative roles, respectively, as a proto-oncogene and an inhibitor of Wnt signaling. We note, however, that our merged list of regulated genes contains several other target genes, directly or indirectly regulated by β-catenin, of potential significance with respect to cell signaling and oncogenesis, including *LMO2*, *ETV5* and *WNT11*. Recently, it has been reported that Wnt-11 promotes transformation of intestinal epithelial cells in culture (Ouko *et al*, 2004). We are currently investigating, using ChIP assays, whether these genes are directly regulated by β-catenin.

The strategy of focusing on genes that appear to be regulated by Wnt/β-catenin signaling in a multiplicity of settings may have limitations; it is increasingly apparent that transcriptional control reflects the interactions of multiple factors and that the abundance of these factors varies among cell types, thereby diversifying the readout of stereotyped signaling pathways. For instance, levels of *c-MYC* RNA are increased by the Wnt/β-catenin pathway in colorectal cancer cells, yet no changes were observed in the systems we used here or in immature $CD34^+$ thymocytes stimulated by activation of the Wnt/β-catenin pathway (Staal *et al*, 2004). However, *LMO2*, one of the genes most affected by Wnt/β-catenin signaling in our cell and tumor systems, is also highly upregulated in thymocytes (Staal *et al*, 2004). In this light, it is probable that important regulatory events will occur uniquely in certain cell types; the challenge will be to identify the crucial events by determining the functional consequences of the many changes in gene expression that result from cell signaling.

Materials and methods

Cell lines and tumor samples

To generate 293Top cells, the kidney embryonic epithelial cell line 293 was first transfected with pcDNA6-*tva* (Fisher *et al*, 1999), a construct expressing the ALV receptor Tva, using stable calcium phosphate (Stratagene) according to the manufacturer's instructions. After selection with 5 μg/ml blasticidin, one clone was chosen for transfection with the β-catenin *luciferase* reporter pOT and a plasmid carrying a neomycin-resistant gene. The pOT reporter consists of three LEF/TCF-binding sites and a minimal promoter driving the *luciferase* gene (Rubinfeld *et al*, 1993). Cells were then selected in 5 μg/ml blasticidin and 200 μg/ml Geneticin (Life Technology, Inc.) and pooled. The rat kidney epithelial cell lines RK3E, RK3E/S33Y-A, RK3E/S33Y-D, and RK3E/DN132-B were kindly provided by Dr Eric Fearon (University of Michigan) and have been described previously (Kolligs *et al*, 1999; Schwartz *et al*, 2003). The ovarian endometrioid tumor sample collection has been described previously (Wu *et al*, 2001). Quick-frozen human colon tumors and normal colon mucosa were obtained from the MSKCC tumor bank collection. Quick-frozen adenomas and normal small intestinal musosa from *Apc^{min/+}* mice were kindly provided by Dr Bert Vogelstein (John Hopkins University).

Viral constructs and infections

The RCAS-GFP virus has been described previously (Holland, 2000). HA-tagged β-*catenin*S37A cDNA was excised from pMH-S37Aβ-*catenin* (Zorn *et al*, 1999) with *Asp*718 and *Apa*1, treated with T4 polymerase to form blunt ends, and cloned into the *Pme*1 site of the ALV retroviral vector RCAS-Y (Dunn *et al*, 2000). To produce recombinant retroviruses, the retroviral constructs were transfected into DF-1 chicken fibroblasts as described previously (Himly *et al*, 1998; Schaefer-Klein *et al*, 1998). To transduce β-*catenin*S37A and *GFP* cDNAs into 293Top cells, supernatants from virus producing DF-1 cells were filtered and diluted 1:1 with fresh

growth media before infecting cells plated at 10% confluency. This process was repeated 24 h later.

Data analysis

To analyze the expression data, we used two complementary methods. Our first approach was to use the MAS 5.1 (Affymetrix) software to perform a cross-comparison of each experimental sample (293TopS37A) with each of the four reference samples (293TopGFP). We selected 2041 genes that demonstrated at least a two-fold difference in expression in one or more of these comparisons (list 1). As an alternative, we utilized the Genespring 6.0 (Silicon Genetics) software package to identify genes differentially expressed between 293TopS37A and 293TopGFP samples, using a parametric test with filtering ($P<0.05$) on variances estimated by cross-gene error model (also known as the Rocke-Lorenzato model; Box *et al*, 1978; Milliken *et al*, 1984). With this second statistical approach, we identified 976 genes that were differentially regulated (list 2). The intersection of lists 1 and 2 yielded 234 regulated genes (list 3). In all, 77 genes in list 3 demonstrated at least two-fold difference in expression levels. U133A-generated microarray data from the OEAs were processed and analyzed as previously reported for data generated using lower density HuGeneFL arrays (Schwartz *et al*, 2003). A list of 563 genes with at least 1.75-fold difference in expression in OEAs with deregulated β-catenin relative to wild-type tumors ($P<0.05$) was obtained.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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