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# Overexpression of Human *Dickkopf-1*, an Antagonist of *wingless/WNT* Signaling, in Human Hepatoblastomas and Wilms' Tumors

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**SUMMARY:** Hepatoblastomas (HBs) represent the most frequent malignant liver tumors of childhood; yet little is known about the molecular pathogenesis and the alterations in expression patterns of these tumors. We used a suppression subtractive hybridization approach to identify new candidate genes that may play a role in HB tumorigenesis. cDNA species derived from corresponding liver and fetal liver were subtracted from HB cDNAs, and a series of interesting candidates were isolated that were differentially expressed. One of the transcripts overexpressed in HB was derived from the human *Dickkopf-1* (*hDkk-1*) gene, which encodes a secreted protein acting as a potent inhibitor of the *wingless/WNT* signaling pathway. We examined the *hDkk-1* expression levels in 32 HB biopsy specimens and in the corresponding liver samples, in 4 HB cell lines, and in a panel of other tumors and normal tissues using a differential PCR approach and Northern blotting. Eighty-one percent of the HBs but none of the normal pediatric or fetal liver tissues showed *hDkk-1* expression. *hDkk-1* transcripts were also present in 5 of 6 Wilms' tumors but only weakly detectable in 2 of 20 hepatocellular carcinoma samples and in 1 of 5 medulloblastoma cell lines; transcripts were absent in malignant gliomas and breast cancer. The central effector molecule in the *WNT* developmental control pathway is the $\beta$-catenin protein. Interestingly, activating mutations of the *$\beta$-catenin* gene have previously been identified in 48% of HBs, and more than 85% of HBs show accumulation of $\beta$-catenin protein as the indicator for an activated pathway. The overexpression of the inhibitor Dkk-1 may therefore be related to uncontrolled *wingless/WNT* signaling and may represent a negative feedback mechanism. *hDkk-1* expression represents a novel marker for HBs and Wilms' tumors. (*Lab Invest 2003, 83:429–434*).

Hepatoblastomas (HBs) represent the most common primary malignant liver tumors of childhood. They are rare tumors with an incidence of about one new case per one million children per year (Mann et al, 1990). Little is known of the molecular pathogenesis of HBs. Cytogenetic and comparative genomic hybridization analyses have demonstrated mainly numeric chromosomal aberrations, with frequent gains of chromosomes 2, 8, and 20 (Weber et al, 2000). Microsatellite analysis revealed regions with allelic loss on chromosomes 1 and 11 (Albrecht et al, 1994; Kraus et al, 1996). Recent studies showed that genes encoding components of the *wingless/WNT* signal transduction pathway, including the *APC*, *AXIN1*, and *$\beta$-catenin* genes, are mutated in a larger subset of sporadic HBs (Koch et al, 1999; Oda et al, 1996; Taniguchi et al, 2002). This may lead to inappropriate activation of the pathway and consecutive induction of growth-promoting genes. The central molecule of this pathway is $\beta$-catenin, which is rapidly degraded in the absence of *Wnt* signaling. The pathway is activated by extracellular Wnt proteins binding to specific ligands of the Frizzled family, leading to $\beta$-catenin stabilization. $\beta$-catenin then enters the nucleus and, together with TCF transcription factors, activates the expression of specific target genes (Polakis, 2000). Pathophysiologic alterations of signaling pathways frequently result in changes in expression levels of transcriptionally regulated target genes. The identification of such dysregulated transcripts may therefore provide clues for the underlying genetic alterations.

In this report we describe the identification and characterization of a differentially expressed developmental control gene, human *Dickkopf-1* (*hDkk-1*), which we identified by means of suppression subtractive hybridization in human HBs (Diatchenko et al, 1996). *hDkk-1* is a member of the *dickkopf* gene family

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and maps to chromosome 10q11.2 (Roessler et al, 2000). *Dkk-1* is expressed in a timely and spatially controlled manner during development. It was first isolated in *Xenopus*, where it is expressed in the Spemann organizer as a head inducer (Glinka et al, 1998). Injection of neutralizing Dkk antibodies leads to microcephaly, and coinjection of Dkk-1 with BMP antagonists into *Xenopus* eggs induces complete head structures (Monaghan et al, 1999). Other members of the family are Dkk-2, Dkk-3, and Dkk-4, which all contain two cysteine-rich domains that are highly conserved among the different family members (Krupnik et al, 1999). Although Dkk-1 functions as an inhibitor of the *wingless/Wnt* signaling pathway (Glinka et al, 1998), Dkk-2 activates *Wnt* signaling in *Xenopus* embryos (Wu et al, 2000). Recent reports have shown that the LDL receptor–related protein 6 (LRP6) functions as a Dkk-1 receptor and that Dkk-1 blocks LRP6-mediated *wingless/Wnt* signaling by blocking Wnt-induced Frizzled-LRP6 complex formation (Bafico et al, 2001; Mao et al, 2001; Semenov et al, 2001). Mao et al (2002) have identified the proteins Kremen1 and Kremen2 as high-affinity Dkk-1 and Dkk-2 receptors; they also determined that Kremen2 forms a complex with Dkk-1 and LRP6, resulting in removal of the Wnt receptor LRP6 from the plasma membrane.

In the present study we describe the identification of *hDkk-1* as a novel molecular marker for HBs and Wilms' tumors (WT) by suppression subtractive hybridization and its expression pattern in primary tumors, cell lines, and normal tissues.

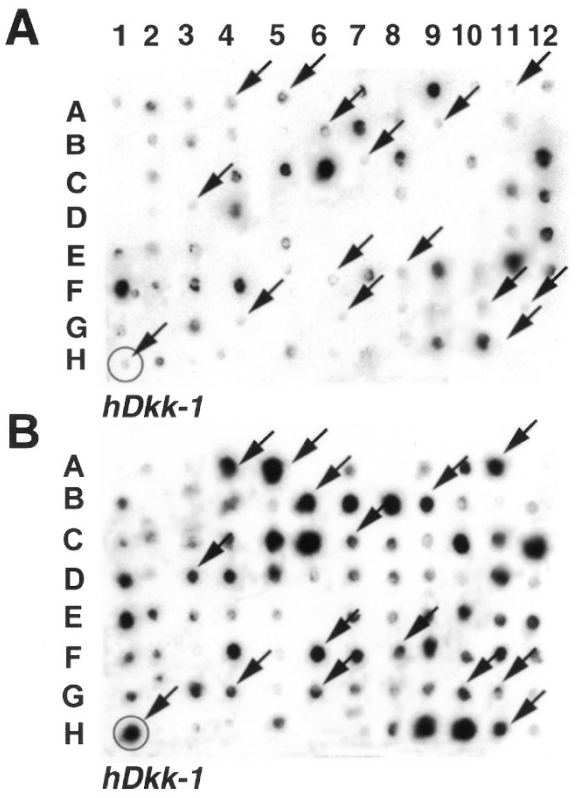


**Figure 1.**
Ninety-six randomly chosen clones of a hepatoblastoma (HB) cDNA library enriched by suppression subtractive hybridization (forward subtraction) were dotted in duplicate on nylon membranes and hybridized with radioisotope-labeled subtracted cDNA libraries from normal/fetal liver (A) and HB (B). The clone corresponding to human *Dickkopf-1* (*hDkk-1*) is marked by a *circle*.

## Results and Discussion

Analysis of differential gene expression patterns in tumors may provide insight into the origin of tumors and the pathomechanisms that underlay tumorigenesis. In addition, these genes may serve as diagnostic and prognostic markers, which can be useful for treatment stratification of the patients (Alizadeh et al, 2000). We used the suppression subtractive hybridization technique for the identification of differentially expressed genes in HBs. Because HB cells resemble immature hepatocytes during embryonal and fetal development, we subtracted cDNA from a patient's liver and cDNA from a fetal liver tissue sample because we intended to identify genes involved in the pathogenesis of HB, but we did not want to select for genes that only reflect an immature stage of liver development. A forward subtraction was performed with the HB tumor sample as "tester" cDNA and the normal and fetal liver as "driver" cDNA; a reverse subtraction was performed with normal samples as tester cDNA and tumor as driver cDNA. The subtracted cDNA libraries were amplified and cloned, and 96 randomly picked colonies of the forward subtraction and 96 colonies of the reverse subtraction were screened for differential expression by dot blot hybridization. A total of 15 of 96 positive clones of the forward subtraction were found to be strongly up-regulated in HBs (Fig. 1). Sequencing and BLAST analysis of these cDNA clones showed homologies to the following genes: *aquaporin 9* (AB008775), *glutamin synthetase* (S70290), *angiotensin II receptor* (Z11162), *α-glucocorticoid-receptor* (X03225), *DEAD-box protein BAT1* (AF029062), *lecithin:cholesterol acetyltransferase LCAT* (X06537), *GTP-binding protein Sara* (BC002847), and *dickkopf-1 Dkk-1* (AB020315). Three clones matched with the mRNA sequence of the HIV-associated non-Hodgkin's lymphoma (HY17171), and two clones corresponded to known EST sequences, KIAA0905 (AB020712) and homo sapiens clone 638 (AF091085). In addition to the genes that have not been previously shown to be differentially expressed in HBs, we identified the *glutamine-synthetase* gene, which is known to be up-regulated in liver cancer (Christa et al, 1994). The re-identification of this gene in our suppression subtractive hybridization approach indicates the efficiency of the subtraction procedure and serves as a positive control. In contrast, 16 of 96 clones were found to be down-regulated in HB. Among these were genes involved in liver metabolism, such as *hemoglobin γ-G*, *hemoglobin γ-A*, *haptoglobin*, and the *fibrinogen β-chain*.

The *hDkk-1* gene that was found to be up-regulated in this screen was of special interest because of its regulatory functions in the *wingless/WNT* pathway. *Wnt* signaling has important implications in embryonic

development and cell differentiation of several organs including the central nervous system, heart, tooth, kidney, palate, and limb buds (Monaghan et al, 1999). Recent studies indicated that Dkk-1 acts as a potent inhibitor of *Wnt* signaling by binding to the transmembrane receptors Krm and LRP5/6, a corepressor of the Wnt/Fz receptor (Mao et al, 2002). *Wnt* signaling is blocked by Dkk-1, and phosphorylated β-catenin becomes a target for ubiquitination and subsequent degradation by the proteasome.

Overexpression of *hDkk-1* in HBs was confirmed by Northern blot analysis (Fig. 2). All tumor samples showed high *hDkk-1* expression, but there was no detectable signal in normal tissues. Furthermore, a large panel of 32 HBs, 18 pediatric liver samples, and 6 fetal livers was examined by differential PCR (Table 1). A total of 26 (81%) of 32 HBs and 5 (100%) of 5 HB cell lines revealed *hDkk-1* expression, whereas none of the liver samples showed transcripts of *hDkk-1* (Fig. 3). This indicates that *hDkk-1* is highly up-regulated in most HBs and that this expression is not simply a feature of immature liver tissue because fetal liver samples were consistently negative.

We have previously shown that the *wingless/WNT* signaling pathway is frequently altered in HBs, and oncogenic *β-catenin* mutations were found in 48% of our cases (Koch et al, 1999). Most mutations were found in the amino-terminal regulatory domain, which is necessary for targeting the β-catenin protein for degradation. Mutation at this site of β-catenin leads to accumulation of β-catenin and activation of the *wingless/Wnt* pathway. These changes may lead to proliferation of liver progenitor cells and thereby may induce HBs. Although 48% of HBs carry mutations in the *β-catenin* gene, more than 85% of HBs show nuclear accumulation of the protein, indicating an activated *WNT* pathway. This suggests that alternative genetic events may occur in other components of the pathway. In addition to *β-catenin*, *Axin1*, *Axin2/Conductin*, and *APC* were found to be altered in a subset of HBs and contribute to the uncontrolled activation of this developmental signaling pathway (Oda et al, 1996; Taniguchi et al, 2002; and our unpublished results). In this series, 25 cases were tested for *β-catenin* mutations. All 13 Hbs carrying a mutation expressed *hDkk-1*, as did 9 of 12 cases without a mutation. Seventeen cases were tested for β-catenin accumulation by immunostaining (Koch et al, 1999). Accumulation was detected in 16 samples, 14 of which also expressed *hDkk-1*.

Interestingly, activating *β-catenin* mutations have been found in both WT and hepatocellular carcinomas (HCC) but in a lower frequency compared with HB (de La Coste et al, 1998; Maiti et al, 2000). We therefore

**Table 1. Summary of the Analysis of *hDkk-1* Expression in Tumors and Normal Tissues by Northern Blotting and RT-PCR**

| Tissue | *hDkk-1*–positive samples/samples tested |
|---|---|
| Hepatoblastoma | 26/32 |
| Hepatoblastoma cell lines | 4/4 |
| Hepatocellular carcinoma | 2/20 |
| Hepatocellular carcinoma cell line | 1/1 |
| Pediatric liver | 0/18 |
| Fetal liver | 0/6 |
| Wilms' tumor | 5/6 |
| Pediatric kidney | 0/2 |
| Breast carcinoma | 0/6 |
| Normal breast tissue | 0/3 |
| Medulloblastoma cell lines | 1/5 |
| Glioblastoma | 0/8 |

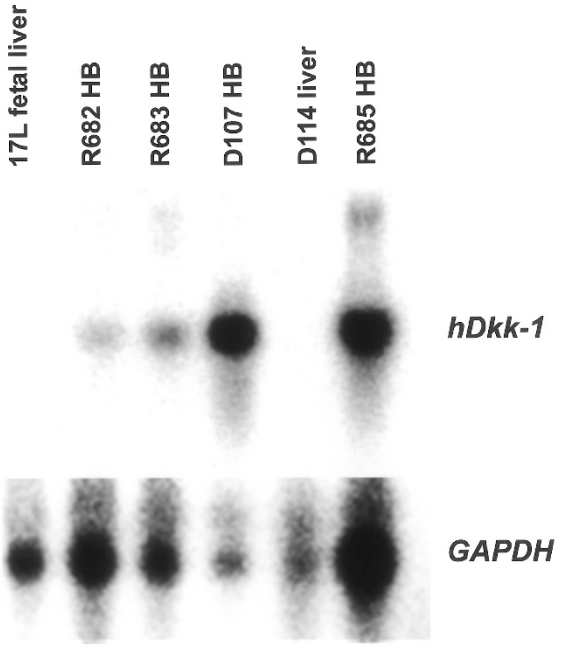


**Figure 2.**
Northern blot analysis of *hDkk-1* expression in HB and liver tissue. Although all four HBs showed a *hDkk-1* signal of approximately 2 kb, no expression was detectable in the normal liver samples (fetal and pediatric). A hybridization with a *GAPDH* probe was used to document RNA loading.

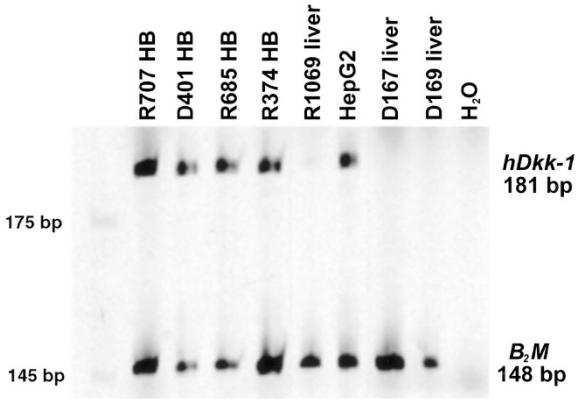


**Figure 3.**
Duplex PCR analysis for *hDkk-1* and *β2-microglobulin* (*β2M*) in hepatoblastoma samples. A specific 181-bp *hDkk*-1 signal was detectable in four HBs and the cell line HepG2 but not in pediatric liver samples. The PCR reaction was shown to be in the exponential phase for both products.

investigated a series of WT, an embryonal malignant childhood tumor of the kidney. Similarly to HB, *hDkk-1* was expressed in five of six WT specimens but was not detected in the adjacent kidney tissue (Fig. 4). We further found *hDkk-1* expression in only 2 of 20 HCC of adult patients, in the HCC cell line HepG2 that is derived from childhood HCC (Fig. 4). *hDkk-1* expression was absent in eight glioblastoma samples, six breast cancer specimens, three normal breast tissues, and four of five medulloblastoma cell lines, the positive exception being the DAOY cell line.

Why is *hDkk-1* highly overexpressed in hepatoblastomas? Recent studies have indicated that the *hDkk-1* gene is a direct target of p53 and contains a p53-responsive element in its promoter (Wang et al, 2000). This finding lead to the hypothesis that Dkk-1 may mediate p53 tumor suppression by its antagonistic effects on the *WNT* signaling pathway (Wang et al, 2000). We therefore analyzed 15 HBs and 5 HB cell lines for p53 expression by immunostaining (data not shown). There was no evidence for p53 overexpression on the protein level. Furthermore we were not able to detect *TP53* mutations in the hot spot region exons 5 to 8 in this series of tumors (data not shown). Therefore, alterations in p53 do not seem to be responsible for *hDkk-1* induction in HBs. Shou et al (2002) demonstrated that DNA damage lead to increased *hDkk-1* mRNA levels in a variety of human tumor cell lines irrespective of their *TP53* gene status, indicating that p53-independent mechanisms also regulate *hDkk-1* expression. These results show that *hDkk-1* harbors proapoptotic potential. In this regard it should be mentioned that most HBs and WTs respond well to chemotherapeutic agents, so that modern treatment protocols include preoperative chemotherapy. Further studies will need to determine whether high *hDkk-1* expression may be associated with a favorable response to treatment.

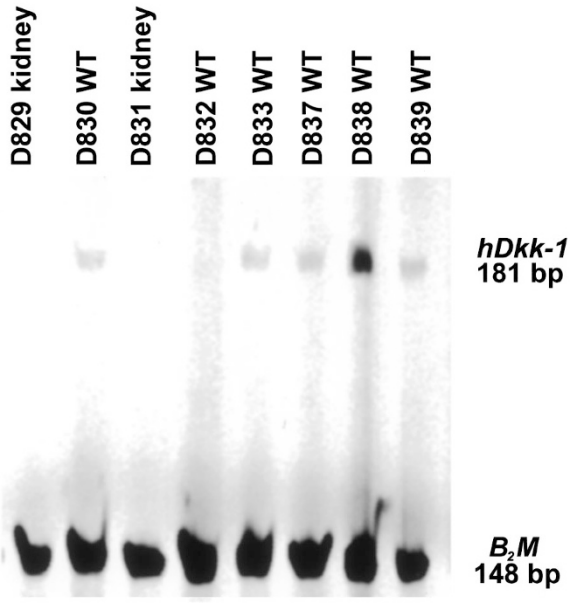


**Figure 4.**
Duplex PCR analysis for *hDkk-1* and $\beta_2$-*microglobulin* ($\beta_2M$) in Wilms' tumor (WT) samples. Five WTs showed *hDkk-1* expression, but no signal was detectable in the pediatric kidney tissues.

The overexpression of *hDkk-1* as an *WNT* inhibitor in HBs and WTs may also represent a negative feedback loop of cells with inappropriately activated *WNT* signaling. We have recently identified a similar feedback loop of the *Axin2/Conductin* gene, which represents a negative regulator of *WNT* signaling and is highly overexpressed in HBs (Lustig et al, 2002). In this case, *Axin2* turned out to be a direct target gene of TCF transcription factors.

In summary we identified the *hDkk-1* gene, a potent inhibitor of the *wingless/WNT* pathway, as a differentially expressed gene in human HB by subtractive suppression hybridization analysis. *hDkk-1* is significantly overexpressed in HBs and WTs and represents a novel molecular marker for these embryonal tumors. A specific function of *hDkk-1* in the pathogenesis of these embryonal neoplasms must be elucidated in further studies.

## Materials and Methods

### *Patients, Tumors, and Cell Lines*

A total of 32 HB biopsy specimens, 4 HB cell lines from sporadic HBs, and 18 normal postnatal and 6 fetal livers (gestational age 13.5 to 18 weeks) were analyzed. The HepT3 hepatoblastoma cell line was derived from tumor D204, and the HepT1 cell line was derived from tumor DZ25 (Pietsch et al, 1996). In these cases we were able to study both the tumor and the derived cell line. We further analyzed the HB cell lines HepT4 and HUH6 (Doi, 1976). The age of the HB patients ranged from 1 to 57 months. For subtractive suppression hybridization we used tumor and corresponding liver from the same HB patient, as well as mRNA from a fetal liver (gestational age 14 weeks).

In addition we tested 20 biopsy samples of HCC and the cell line HepG2 (Aden et al, 1979), which was derived from a pediatric HCC. We further studied six WT and two corresponding normal kidney tissues, five medulloblastoma cell lines (MHH-MED-1, MHH-MED-3, MHH-MED-4 [Pietsch et al, 1994], D341-Med, Daoy), eight glioblastoma samples, six breast cancer biopsy samples, and three normal breast tissues.

### *RNA Preparation and cDNA Synthesis*

Before RNA extraction, individual tissue samples were preexamined by frozen section histologic examination to document the histopathologic appearance of the specimen. Total cellular RNA was prepared by lysis in guanidinium isocyanate and ultracentrifugation through a cesium chloride cushion (Sambrook et al, 1989) or with the Trizol reagent (Invitrogen, San Diego, California), according to the protocol of the supplier. Contaminating residual genomic DNA was removed by digestion with RNAse-free DNAse (Boehringer Mannheim, Indianapolis, Indiana) before reverse transcription, which was performed using the Superscript Preamplification System (Invitrogen) with random hexamers as primers.

### *Suppression Subtractive Hybridization*

For subtractive hybridization, poly($A^+$) mRNA was purified by poly(T) chromatography (Oligotex columns; Qiagen, Studio City, California) from total RNA of HB and liver tissue from the same patient. This was a male patient who underwent surgery without prior chemotherapy and had an HB with epithelial histology. We pooled the liver RNA with poly($A^+$) mRNA from a fetal liver. Subtractive hybridization was performed according to the protocol of the manufacturer (PCR-select cDNA subtraction kit; Clontech Laboratories, Heidelberg, Germany). One microgram of each type of mRNA was reverse transcribed at 42° C for 1 hour using Maloney murine leukemia virus reverse transcriptase and a cDNA synthesis primer (5'-$TTTTGTACAAGCTT_{30}$-3') provided in the kit. The cDNAs of the tumor and the normal tissues were digested with *Rsa*I to yield shorter blunt end fragments. The population of the tumor and normal tissue cDNA fragments was separated into two portions, and each portion was ligated to a different adaptor. After subtractive hybridization the entire population of molecules was used in a suppression PCR with the primers and PCR conditions recommended by the provider. All PCR and hybridization steps were performed in a Perkin Elmer GeneAmp 9600 thermal cycler (Norwalk, Connecticut). A forward subtraction with tumor tissue as tester cDNA and normal tissue as driver cDNA and a reverse subtraction with normal tissue as tester cDNA and tumor tissue as driver cDNA were performed on a PE Gene Amp 9600 thermal cycler. The amplified fragments were cloned into a plasmid vector pCR2.1 using the TOPO TA cloning kit (Invitrogen), and ligation products were electroporated into ElectroMAX DH10B *Escherichia coli*–competent cells (Invitrogen).

### *Screening for Differential Expression*

Ninety-six randomly picked clones from the forward subtraction and 96 clones from the reverse subtraction were screened for differential expression between tumor and normal tissue with a dot blot assay using the protocol of the manufacturer (PCR-select cDNA differential screening kit; Clontech, Palo Alto, California). Five microliters of amplified product of each clone was mixed with 5 $\mu$l of 0.6 N NaOH, spotted on duplicate nylon membranes (Hybond $N^+$; Amersham Biosciences, Freiburg, Germany), and cross-linked (Stratalinker; Stratagene, La Jolla, California). We used $^{32}$P-labeled probes from the tester (hepatoblastoma) and driver (liver) subtracted cDNA libraries for dot blot hybridization.

### *DNA Sequencing*

The clones that were confirmed to be differentially expressed in the dot blot assay were sequenced using the Fluorescent Dideoxy Terminator Kit (Applied Biosystems, Foster City, California). The reaction products were analyzed on an ABI373A DNA-Sequencer (Applied Biosystems) using the Sequence Editor 1.0.3 Software (Applied Biosystems). The BLAST-Database (http://www.ncbi.nlm.nih.gov/BLAST) was screened for matching sequences.

### *Northern Blot Analysis*

To confirm differential expression after the screening step, we performed Northern blots with RNA samples of HB biopsy specimens and pediatric and fetal liver tissues. For Northern blot analysis, 15 $\mu$g of total cellular RNA were separated on 1% formaldehyde denatured agarose gels, blotted to Hybond-$N^+$ membranes (Amersham), and cross-linked. For *hDkk*-1 mRNA detection, a 390-bp PCR fragment (base 466 to 856, GenBank accession number AB020315) was used as a probe and labeled with $^{32}$P using the Strip-Ez DNA kit (Ambion, Huntingdon, United Kingdom). Variations of RNA loading and RNA integrity were checked by hybridization to a human GAPDH 452-bp cDNA probe (bases 601 to 1052, accession number NM002046).

### *RT-PCR-Analysis of* hDkk-1 *Expression*

Analysis of the *hDkk-1* mRNA expression was performed by a sensitive differential reverse transcription-PCR assay using *hDkk-1*–specific primers together with primers for the housekeeping-gene $\beta_2$-*microglobulin*. Primer sequences were as follows: *hDkk-1*, 5'-GGTATTCCAGAAGAACCACC-3' and 5'-GAGAGCCTTTTCTCCTATGC-3', product size 181 bp; and $\beta_2$-*microglobulin*, 5'-TGTCTTTCAGCAAGGACTGG-3' and 5'-GATGCTGCTTACATGTCTCG-3', product size 148 bp. One of the primers for each gene was labeled with a fluorescent dye. The primers were chosen from adjacent exons spanning intronic sequences to avoid signals of the cDNA product size caused by residual genomic DNA. The PCR protocol consisted of an initial denaturation step of 94° C for 5 minutes, followed by 30 cycles of a three-step program of 94° C for 30 seconds, 58° C for 40 seconds, and 72° C for 35 seconds, and a final extension step of 72° C for 10 minutes. The PCR was performed on a Biometra Uno thermo-cycler in a final volume of 10 $\mu$l in the presence of 1.5 mM $MgCl_2$, 0.25 U of *Taq* polymerase in PCR buffer (all from Invitrogen), and 10 pmol of the $\beta_2$-*microglobulin* and 25 pmol of the *hDkk-1* primers. PCR products were separated and analyzed on 6% polyacrylamide gels using a LI-COR L-4200 DNA sequencer (MWG-Biotech).

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