Murine Nr4a1 and Herpud1 are up-regulated by Wnt-1, but the homologous human genes are independent from β-catenin activation

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The Wnt signal transduction pathway regulates morphogenesis and mitogenesis of cells in multicellular organisms. A major downstream consequence of Wnt-1 signalling is the activation of β-catenin/T-cell factor (TCF)-mediated transcription. We compared Wnt-1-transformed murine mammary epithelial cells with control cells by subtractive hybridization. We found the two genes Nr4a1 and Herpud1 to be overexpressed in Wnt-1-transformed cells. Remarkably, the transcription levels of the two homologous human genes NR4A1 and HERPUD1 are neither activated in cells with activated β-catenin/TCF-mediated transcription nor can be induced by β-catenin transfection. These results indicate different regulation mechanisms of the two genes in murine and human cells.

Key words: C57MG/Wnt-1 cell, Mif1, nur77, suppression subtractive hybridization, Wnt target gene.

INTRODUCTION

Members of the Wnt protein superfamily are secreted glycoproteins that regulate cell growth, differentiation, organogenesis and oncogenesis (for a review see [1]) [2,3]. By binding to a membrane receptor of the Frizzled family, Wnt activates an intracellular pathway that leads to the inactivation of the glycogen synthase kinase 3β (GSK3β). In the absence of the Wnt signal, GSK3β phosphorylates the proto-oncoprotein β-catenin that targets it for ubiquitin-mediated degradation by a multiprotein complex (for a review see [4]). This complex includes GSK3β, the tumour-suppressor protein adenomatous polyposis coli (APC), Axin and a member of the SCF (Skp1-Cull-F box) ubiquitin ligase complex, β-TrCP/Slimb [5]. In the presence of the Wnt signal, the β-catenin level rises in the cytoplasm, reaching a critical amount that enables it to bind to a protein of the T-cell factor (TCF)/Lef family of transcription factors [6,7]. This complex translocates to the nucleus, where it binds to DNA and activates the transcription of specific target genes [8]. Several genes activated by the Wnt signal have been identified, including connexin 43, cyclo-oxygenase-2, the Wnt-1-inducible signalling pathway protein (WISP) genes and Wrch-1 [9–12]. In addition, many genes have been identified to be up-regulated by activation of β-catenin or by inactivation of APC, respectively. These include the proto-oncogene c-*myc*, the transcription factors peroxisome proliferator-activated receptor δ ('PPARδ'), c-*jun* and fra-1, the cell-cycle-regulator protein cyclin D1, the protease matrilysin and the β-catenin-down-regulating protein axin2 [13–17].

Recent studies revealed that the Wnt signalling pathway represents a multifaceted pathway, which includes branching points and feedback loops on several levels, rather than a single linear hierarchic cascade. First, body-axis determination in *Xenopus* during embryonic development is regulated by genes that are controlled by the β-catenin activation of Lef/TCF proteins but which are independent of the Wnt signal (for a review see [18]). The independence of β-catenin activation and Wnt signalling was also confirmed by the transfection of murine cells with oncogenic Wnt-1, which led to morphological and proliferative responses, which could not be induced by transfection of an active β-catenin mutant [19]. Indeed, the murine homologue of the human basic transcription element-binding protein 2 (BTEB2) transcription factor has been identified as a target gene which is responsive to Wnt-1 but independent of β-catenin activation [20]. Furthermore, a negative feedback loop was identified by the finding that the negative Wnt signalling regulator axin2 is up-regulated in colorectal and liver tumours [21,22].

In order to identify novel Wnt-1-regulated genes, we used the method of cDNA suppression subtractive hybridization to compare RNA from two cell lines: C57MG/Wnt-1 cells, which are murine mammary epithelial cells that have been transformed by stable transfection with the retroviral vector containing the Wnt-1 gene, and C57MG/PLNCx cells, which have been derived from the corresponding C57MG mother cell line by transfection with empty vector [8].

MATERIALS AND METHODS

Cell culture

The colorectal tumour cell lines HCT116 and SW480 and the colon normal cell line CCD841CoN were purchased from American Type Culture Collection. The cell lines were cultured under recommended conditions [23,24]. Total RNA from the human colorectal normal cell line NCM460 was purchased from InCell Corporation (San Antonio, TX, U.S.A.) [25]. Murine mammary epithelial C57MG cells [26] were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/l glucose, 10 mg/l insulin and 10 % fetal bovine serum. Retroviral transfection with Wnt-1 and morphological features of the Wnt-1-transformed cells have been described in [8]. In addition to morphological features of transformed cells, the C57MG/Wnt-1 cells show a high level of β-catenin as a result of stable Wnt-1

Abbreviations used: APC, adenomatous polyposis coli; GSK3β, glycogen synthase kinase 3β; TCF, T-cell factor.

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expression. We compared the expression profile of Wnt-1-transfected C57MG cells with the profile of C57MG/PLNCx cells. C57MG/PCLNx cells are cells of the parent cell line C57MG that have been transfected with empty vector and have undergone the same selection process as C57MG/Wnt-1 cells.

TS/A murine mammary adenocarcinoma cells [27] were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10 % fetal calf serum. For tumour formation 10^6 TS/A cells were injected subcutaneously into female SCID/beige mice (Harlan). Animals were killed at a tumour size of about 1.5 cm in diameter, and tumours were excised, fixed and analysed [28].

Suppression subtractive hybridization

We screened for Wnt-1-up-regulated genes by a PCR-based cDNA suppression subtraction hybridization technique [29]. Total RNA was purified from confluent cell layers and incubated with RNase-free DNase to avoid unspecific results from genomic DNA. cDNA was synthesized from 2 μg of mRNA with avian myeloblastosis virus reverse transcriptase for first-strand synthesis and T4 DNA polymerase for second-strand synthesis. Restriction, adaptor ligation and hybridization steps were performed as described [29]. The differentially transcribed gene sequences were PCR-amplified, ligated into pCR2.1 (Invitrogen), cloned and sequenced.

Reverse Northern and Northern blot analysis

Reverse Northern and Northern blot analysis were performed to confirm the differential expression and to analyse the expression patterns of the identified genes. The candidate gene sequences were amplified using plasmid DNA containing the cloned cDNAs of the candidates as templates. For reverse Northern equal amounts of each PCR product were transferred directly on to a pre-wetted nylon membrane using a dot-blot apparatus (Bio-Rad). After washing, the membrane was dried and the immobilized DNA was cross-linked with UV light. Total RNA from C57MG/PCLNx and C57MG/Wnt-1 cells was reverse transcribed into radioactively labelled cDNA using poly(dT)-primed reverse transcription in the presence of [α-^{32}P]dCTP. Non-incorporated nucleotides were removed using a PCR fragment purification kit (Qiagen). Radioactivities of the labelled cDNAs were determined by liquid scintillation counting. After heat denaturation, aliquots of the cDNAs representing equal amounts of radioactivity were added to each membrane. The differentially expressed candidate genes that could be confirmed by reverse Northern blotting were analysed by conventional Northern blotting. After denaturing agarose gel electrophoresis, the total RNA was blotted on to nitrocellulose by capillary transfer. To analyse the tissue- and tumour-specific expression patterns of the identified genes, pre-made multiple-tissue expression arrays were hybridized (Clontech Laboratories). The cDNA fragments of murine and human homologues of the genes Herpud1 or Nr4a1 were [α-^{32}P]dCTP-labelled by cyclic labelling or by random-primed labelling. Hybridizations of reverse Northern and Northern hybridizations were done according to standard protocols.

Transfection and immunocytochemical detection

To analyse the induction of transcription, the β-catenin sequence was PCR-mutagenized to the transcription-activating mutant Ser-33→Tyr (S33Y). This sequence was cloned into the mammalian expression vector pCI-neo (Promega) and transfected into HEK-293 cells with transfection detergent (Qiagen). The transcription levels of HERPUD1 and NR4A1 were analysed by Northern blot and compared with the levels in cells transfected with empty vector. The intracellular localization of the HERPUD1 protein was analysed by fluorescence microscopy of HEK-293 cells that had been transfected with the HERPUD1 gene fused to the gene for green fluorescent protein. The intracellular localization of the Nr4a1 protein was analysed by immunostaining C57MG/Wnt-1 cells with primary polyclonal anti-Nr4a1 antibody (Santa Cruz Biotechnology) and a secondary Cy3-labelled donkey anti-goat antibody.

Reporter gene assay

Reporter gene assays were performed as described in [30,31]. Rabbit kidney epithelial-like RK13 cells were grown to 40–70 % confluence on 6 cm dishes and then transfected with a total of 10 μg of DNA by the calcium phosphate co-precipitation method. We used the following vectors and constructs where indicated: pKS+/Ltk80-luc (control vector), topflash motif in pKS+/Ltk80-luc, the Nr4a1 promotor in pKS+/Ltk80-luc, the β-galactosidase expression vector pEQ176 and β-catenin S33Y in pCI-neo. The topflash promoter is a β-catenin/TCF-dependent promoter consisting of the repetitive TCF-binding site WWCAAAG [31]. We cloned a synthetic oligonucleotide spanning four consecutive motifs in the pKS+/Ltk80-luc vector. This luciferase reporter gene under the control of the topflash promoter was used as a positive control. For lithium stimulation experiments cells were incubated with 20 mM LiCl for 24 h. After transfection (36 h) cells were harvested and lysed. Luciferase and β-galactosidase activities were determined and relative luciferase activity was obtained by normalizing luminescence to β-galactosidase activity.

Accession numbers

The GenBank accession numbers of the nucleotide sequences used are: Herpud1, NM022331; HERPUD1, XM027736; Nr4a1, NM010444; NR4A1, D85245.

RESULTS

The transcription levels of Nr4a1 and Herpud1 are elevated in Wnt-1-transformed C57MG cells

After cloning the library of differentially amplified sequence fragments, 192 single colonies were picked, the plasmids were purified and the inserts were amplified using flanking primers. For reverse Northern analysis equivalent amounts of each PCR fragment were immobilized on membranes and hybridized with a cDNA library from C57MG/PLNCx or C57MG/Wnt-1 cells, respectively (results not shown). A fragment was regarded as a putative candidate when it showed at least a 2-fold stronger signal on the membrane hybridized with cDNA from C57MG/Wnt-1 cells compared with the corresponding signal on the membrane hybridized with control cDNA from C57MG/PLNCx cells. The mRNA levels of seven candidates were analysed by conventional Northern blot. The evaluation of the mRNA levels by Northern blot revealed the increased transcription levels of two genes in C57MG/Wnt-1 cells compared with C57MG/PLNCx cells (Figure 1). First, we detected a 400 bp sequence fragment, which is identical with a fragment of the gene Nr4a1, formerly called nur77, which is the murine homologue of the human TR3 orphan receptor [32–34]. Secondly, we identified a 443 bp gene fragment, which turned out to be a fragment of the murine homologous gene of human HERPUD1 ('homocysteine-inducible, endoplasmic reticulum, stress-inducible, ubiquitin-like domain member 1'), also called Mif1, which was originally cloned as KIAA0025 in a sequencing project [35,36].

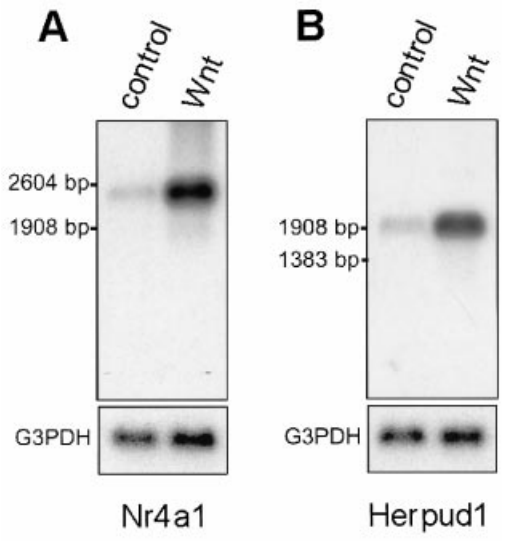

Figure 1 The transcription of Nr4a1 and Herpud1 is activated in Wnt-1-transfected C57MG/Wnt-1 cells (Wnt) compared with C57MG/PLNCx cells transfected with control vector (control)

Northern blots of total RNA were probed with a ^{32}P-labelled fragment of Nr4a1 (**A**) or Herpud1 (**B**). To show the equal loading of RNA, the membranes were also hybridized with a ^{32}P-labelled probe of the ubiquitously expressed gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH).

The transcription of Nr4a1 is induced by lithium and the Nr4a1 promoter is activated by lithium and β-catenin

Lithium ions inhibit GSK3β and increase the nuclear β-catenin protein level, which leads to the activation of β-catenin/TCF-induced transcription. Lithium is thus able to mimic Wnt signalling [37]. To test whether Nr4a1 transcription is activated by lithium, we analysed lithium-treated C57MG cells for the transcription level of Nr4a1. We found an elevated level in lithium-treated cells in comparison with untreated cells (Figure 2A). Therefore one could assume that the promoter of a Wnt-responsive gene is activated indirectly by lithium and directly by β-catenin/TCF. We cloned the promoter region of Nr4a1 and analysed the region for the responsiveness to lithium in a reporter gene assay. The addition of lithium to the assay activates the Nr4a1 promoter 2-fold (Figure 2B). To test whether the Nr4a1 promoter is activated by β-catenin, we analysed the activity of the promoter in the reporter gene assay after transfection of the constitutively active S33Y mutant of β-catenin. We found a 2-fold activation in β-catenin S33Y-transfected cells (Figure 2C). Since lithium mimics Wnt signalling by GSK3β inhibition and Wnt signalling is transferred via β-catenin, these results confirm that Nr4a1 is a target gene of the Wnt pathway.

The Nr4a1 protein is localized in the nucleus independent from Wnt-1 transformation and tumour progression

In human cancer cells, apoptotic stimuli lead to the activation of NR4A1 and the relocalization of the protein from the nucleus to the mitochondria, where it induces cytochrome *c* release [38]. Mitochondrial targeting of NR4A1, but not its nuclear DNA binding and transactivation, is essential for its pro-apoptotic effect. To get an idea about the intracellular localization of murine Nr4a1 in Wnt-1-stimulated cells, we analysed C57MG/Wnt-1 cells and sections from murine mammary tumours by immunocytochemical analysis. Remarkably, the Nr4a1 protein was found selectively in the cell nuclei in all analysed cell types: Nr4a1 showed nuclear localization in C57MG cells whether the cells were Wnt-1-transfected or not (Figure 3A). The nuclear localization was also found in the cells from murine mammary tumours (Figure 3B). Thus the intracellular localization of the Nr4a1 protein seems to be independent from Wnt-1 transformation or tumour progression.

Regarding the intracellular localization of the Herpud1 protein, we confirmed recent findings [35]. We found the protein localized around the nucleus and co-localized with the endoplasmic reticulum (results not shown).

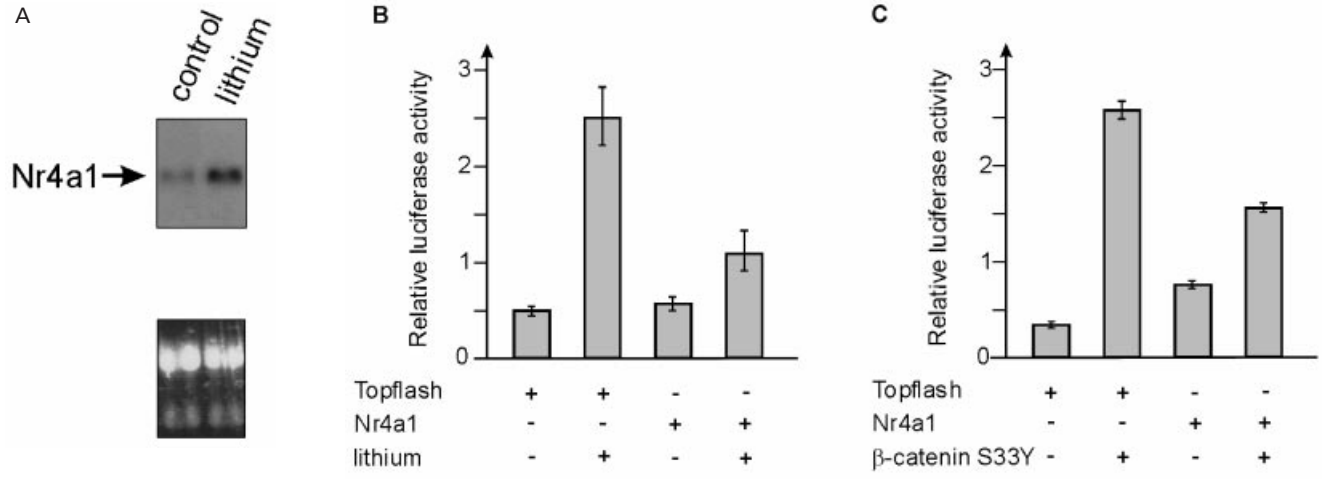

Figure 2 The transcription of Nr4a1 is activated by lithium and the Nr4a1 promoter is activated by lithium and by constitutively active β-catenin

(**A**) Total RNA from C57MG cells (control) and from C57MG cells that had been treated with LiCl (lithium) were probed with Nr4a1. The ethidium bromide-stained agarose gel is shown to demonstrate equal loading. (**B**) Luciferase reporter gene assays were performed to measure the Nr4a1 promoter activity. The Nr4a1 promoter shows 2-fold activation in the presence of lithium. As a control, the activation of the β-catenin/TCF-responsive topflash promoter by lithium is shown. (**C**) Luciferase reporter gene assay to measure the Nr4a1 promoter activity. The Nr4a1 promoter shows 2-fold activation in cells transfected with the constitutively active mutant β-catenin S33Y. As a control, the activation of the β-catenin/TCF-responsive topflash promoter by β-catenin S33Y is shown.

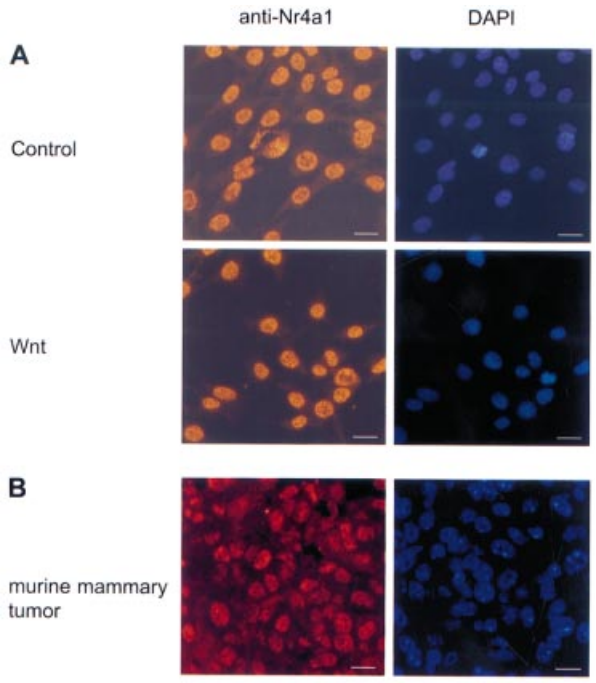

Figure 3 The Nr4a1 protein is located in the cell nuclei

Cultured cells (**A**) or tumour sections (**B**) were immunostained with anti-Nr4a1 antibody as primary antibody (left column) and co-stained with the DNA dye DAPI (4,6-diamidino-2-phenylindole) to visualize the nuclei (right column). (**A**) The Nr4a1 protein is localized in the nuclei of C57MG cells transfected with empty vector (Control) or Wnt-1 (Wnt). (**B**) In murine mammary tumour cells the Nr4a1 protein is localized in the cell nuclei. Scale bars, 20 μm.

Transcription of the human genes NR4A1 and HERPUD1 is not induced in APC-inactivated or β-catenin-activated human cells

Next we wanted to know whether the results obtained with the murine genes might be transferable to the homologous human genes NR4A1 and HERPUD1. Since APC inactivation or β-catenin activation have similar effects to the Wnt-1 signal, we speculated that the expression levels of NR4A1 and HERPUD1 are up-regulated in cells with an inactivating mutation in the APC gene or an activating mutation in the β-catenin gene. To test this hypothesis, we chose the colorectal cancer cell lines SW480 and HCT116, which carry APC-inactivating and β-catenin-activating mutations, respectively [39,40]. We compared the transcription levels of the genes NR4A1 and HERPUD1 in these cell lines with the transcription levels in the normal human colorectal cell lines NCM460 and CCD841CoN, respectively. NR4A1 showed slightly lower transcription levels in the cell lines SW480 and HCT116 from colorectal carcinomas compared with the two cell lines from normal colorectal tissue NCM460 and CCD841CoN (Figure 4). HERPUD1 showed lower transcription in HCT116 cells compared with NCM460 cells. In addition, we analysed the mRNA levels of the genes NR4A1 and HERPUD1 in HEK-293 cells, after transient transfection with the oncogenic β-catenin mutant (S33Y). We did not detect any differences in the expression levels of the two genes in β-catenin-transfected cells compared with cells transfected with empty vector (Figure 4). As a control experiment we compared the transcription levels of the β-catenin/TCF-responsive gene cyclin D1 in the two normal cell lines with the level in SW480 cells. As expected, the cyclin D1 transcription level was elevated in the tumour cell line.

The genes NR4A1 and HERPUD1 are expressed ubiquitously in human tissues; NR4A1 shows lower expression levels in most tumours than in normal tissues

Using multiple tissue expression arrays, we analysed the tissue-specific transcription patterns of the two human genes NR4A1 and HERPUD1. Both genes showed signals of similar intensities in all tissues (results not shown). The NR4A1 gene shows only little higher transcription levels in samples from myogenic tissues and from endocrine glands compared with other tissues. HERPUD1 shows slightly higher transcription levels in samples from particular regions of the brain in comparison with other tissues.

By comparing the transcription levels in 68 human tumours from different tissues, we found that NR4A1 showed higher transcription levels in three tumours and lower transcription

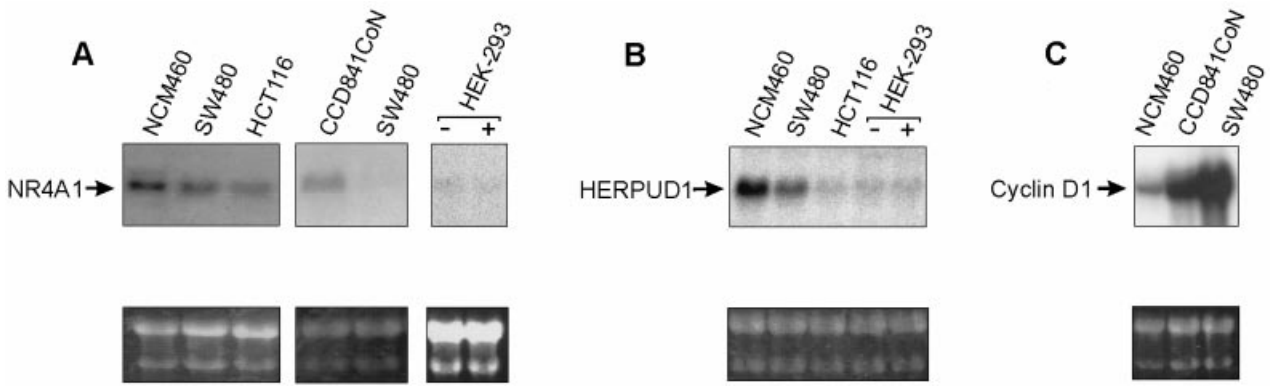

Figure 4 The transcription levels of the human genes NR4A1 and HERPUD1 are not increased in tumour cells and not increased by constitutively active β-catenin S33Y mutant

The transcription levels of NR4A1 (**A**) or HERPUD1 (**B**) were analysed by Northern blots of total RNA from the two normal colon mucosa cell lines NCM460 and CCD841CoN and the two colon carcinoma cell lines SW480 and HCT116, respectively. In both carcinoma cell lines the APC/β-catenin pathway is constitutively activated due to mutations in the APC (SW480) or β-catenin genes (HCT116). Interestingly, the transcription levels of NR4A1 and HERPUD1 are lower in the two tumour cell lines compared with the normal cell lines. Additionally, HEK-293 cells transfected with the constitutively active β-catenin S33Y mutant (+) do not show increased transcription levels of the two genes in comparison to cells transfected with empty vector (−). The ethidium bromide-stained agarose gels are shown to demonstrate equal amounts of loading. (**C**) Control Northern blot to show the increased transcription level of the β-catenin/TCF-responsive gene cyclin D1 in SW480 cells in comparison with the transcription levels in NCM460 or CCD841CoN cells.

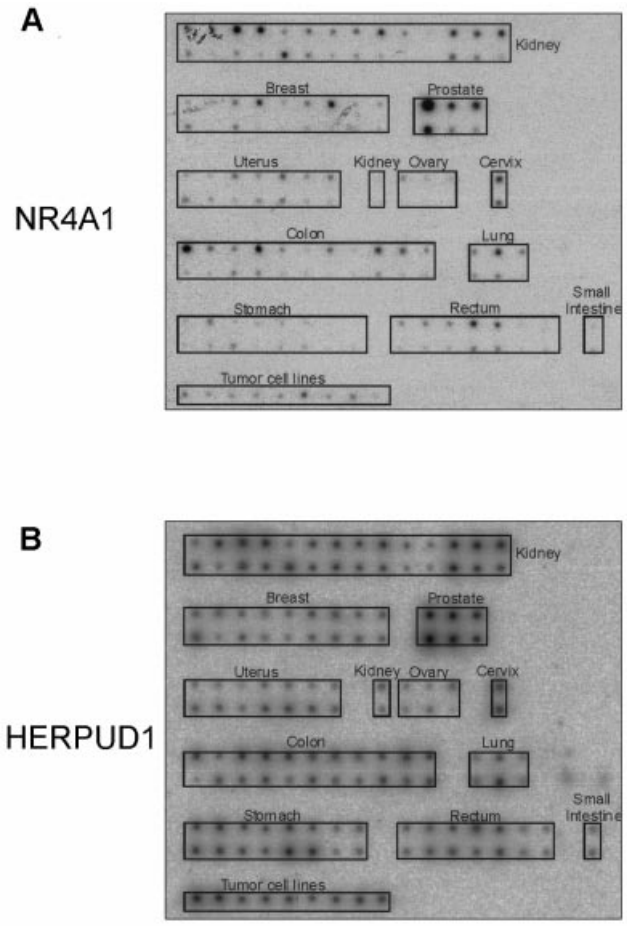

Figure 5 Pre-made expression arrays of 68 human tumours from different tissues and of corresponding normal tissues were probed with NR4A1 (A) or HERPUD1 (B)

The matching tumour and normal tissues are plotted in two parallel rows, whereby the upper rows represent the normal tissues, and the lower rows represent the tumours. (**A**) The NR4A1 transcription level is lower in most human tumours compared with the corresponding normal tissues. Remarkably, the transcription levels of NR4A1 were found to be lower in 33 of 68 tumours compared with the levels in the matching normal tissues. Only three tumours (one each from kidney, uterus and stomach) showed higher transcription levels than the normal tissues. (**B**) The HERPUD1 gene shows similar transcription levels in most tumours and normal tissues. Seven tumours showed higher transcription levels (one from kidney and breast, five from uterus), and six tumours showed lower transcription levels of HERPUD1 compared with the corresponding normal tissues (three from kidney, two from colon, one from breast).

levels in 33 tumours compared with the corresponding normal tissue (Figure 5). In the case of HERPUD1, seven tumours showed higher transcription levels, and six tumours showed lower transcription levels compared with the corresponding normal tissues.

DISCUSSION

An exact knowledge of the genes that are controlled by the Wnt pathway would enhance our understanding of the regulation of cell division and differentiation. These genes might serve as novel targets for anti-cancer therapy or as parameters for analysing embryonic development. Here we provide data showing that the two murine genes Herpud1 and Nr4a1 are targets of Wnt-1 activation. Our findings are based on data from different experimental levels. (i) The genes were identified by subtractive hybridization, when Wnt-1-transfected C57MG cells were compared with corresponding cells transfected with empty vector. (ii) The levels of the two gene transcripts are higher in Wnt-1-transfected cells compared with control cells. (iii) Incubation of the cells with lithium, which inhibits GSK3β analogously to Wnt-1, increases the Nr4a1 transcript level. (iv) The Nr4a1 promoter is activated by lithium and by a constitutively active mutant of β-catenin. Since the localization of the Herpud1 promoter is not yet known, we did not perform reporter gene assays with Herpud1.

Remarkably, the two human genes NR4A1 and HERPUD1 are not up-regulated in cultured tumour cells with inactivated APC or activated β-catenin. Additionally, the transcription levels of the two genes are similar or even lower in most human tumours compared with the corresponding normal tissues. We could not detect significant levels of the NR4A1 protein in sections from human colorectal tumours (results not shown). These findings correspond to the results showing that, in contrast with the transcription level of the murine Nr4a1 gene, the transcription levels of the two human genes NR4A1 and HERPUD1 are not up-regulated by transfection with constitutively active β-catenin. These results were unexpected, since most Wnt-1-responsive target genes reported so far respond to Wnt-1 through activation of the β-catenin–Lef/TCF transcription activation complex and are thus up-regulated in cells with inactivated APC or activated β-catenin (for an overview of Wnt responsive genes see http://www.stanford.edu/%7Ernusse/wntwindow.html). However, there is already an example of a Wnt-1-responsive gene with decreased transcription level in human tumours [11]. There are two possible explanations for the finding that the Wnt responsiveness of a murine gene does not necessarily include its up-regulation in human tumour cells with inactivated APC or activated β-catenin. First, the regulation mechanism of the murine genes Nr4a1 and Herpud1 might be different from the regulation of the homologous human genes. Secondly, the two genes might be new members of this group of genes, and in fact be Wnt-1-responsive but β-catenin/TCF-4-independent [20]. In the case of Nr4a1, the second explanation is not very likely, since its promoter can be induced by constitutively active β-catenin.

NR4A1 has dual intracellular roles. First, NR4A1 is an immediate early gene induced by growth factors. The nuclear NR4A1 protein forms heterodimers with the retinoid X receptor and activates gene transcription [41]. Secondly, apoptotic stimuli may lead to the increase of the NR4A1 protein level in the cytosol, where it associates with mitochondria resulting in the release of cytochrome *c* and cell death [38]. We found the murine homologue Nr4a1 in Wnt-1-transfected cells and also in mammary tumour cells mainly localized in the nuclei, which implies a role in the activation of transcription and of proliferation. Wnt-1 transformation does not influence the nuclear localization of Nr4a1. Considering our finding that Nr4a1 transcription is induced by Wnt-1, one could assume that Nr4a1 acts as a Wnt-1-inducible transcription activator. The Nr4a1 receptor is able to bind to the long terminal repeat of the mouse mammary tumour virus, which might lead to the activation of the virus [42]. Proviral integration of mouse mammary tumour virus into the genome of murine breast epithelial cells leads to the activation of Wnt-1 [43]. Since we identified Nr4a1 as a Wnt-1-responsive gene, our findings suggest an autocrine activation loop resulting from mouse mammary tumour virus infection.

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REFERENCES

1 Dale, T. C. (1998) Signal transduction by the Wnt family of ligands. Biochem. J. **329**, 209–223
2 Nusse, R. (1999) WNT targets. Repression and activation. Trends Genet. **15**, 1–3
3 Bienz, M. and Clevers, H. (2000) Linking colorectal cancer to Wnt signaling. Cell **103**, 311–320
4 Seidensticker, M. J. and Behrens, J. (2000) Biochemical interactions in the wnt pathway. Biochim. Biophys. Acta **1495**, 168–182
5 Kitagawa, M., Hatakeyama, S., Shirane, M., Matsumoto, M., Ishida, N., Hattori, K., Nakamichi, I., Kikuchi, A., Nakayama, K. and Nakayama, K. (1999) An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. EMBO J. **18**, 2401–2410
6 Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996) Functional interaction of β-catenin with the transcription factor LEF-1. Nature (London) **382**, 638–642
7 Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O. and Clevers, H. (1996) XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. Cell **86**, 391–399
8 Shimizu, H., Julius, M. A., Giarre, M., Zheng, Z., Brown, A. M. and Kitajewski, J. (1997) Transformation by Wnt family proteins correlates with regulation of β-catenin. Cell Growth Differ. **8**, 1349–1358
9 van der Heyden, M. A., Rook, M. B., Hermans, M. M., Rijksen, G., Boonstra, J., Defize, L. H. and Destree, O. H. (1998) Identification of connexin 43 as a functional target for Wnt signalling. J. Cell Sci. **111**, 1741–1749
10 Howe, L. R., Subbaramaiah, K., Chung, W. J., Dannenberg, A. J. and Brown, A. M. (1999) Transcriptional activation of cyclooxygenase-2 in Wnt-1-transformed mouse mammary epithelial cells. Cancer Res. **59**, 1572–1577
11 Pennica, D., Swanson, T. A., Welsh, J. W., Roy, M. A., Lawrence, D. A., Lee, J., Brush, J., Taneyhill, L. A., Deuel, B., Lew, M. et al. (1998) WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. Proc. Natl. Acad. Sci. U.S.A **95**, 14717–14722
12 Tao, W., Pennica, D., Xu, L., Kalejta, R. F. and Levine, A. J. (2001) Wrch-1, a novel member of the Rho gene family that is regulated by Wnt-1. Genes Dev. **15**, 1796–1807
13 He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998) Identification of c-MYC as a target of the APC pathway. Science **281**, 1509–1512
14 He, T. C., Chan, T. A., Vogelstein, B. and Kinzler, K. W. (1999) PPARδ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. Cell **99**, 335–345
15 Mann, B., Gelos, M., Siedow, A., Hanski, M. L., Gratchev, A., Ilyas, M., Bodmer, W. F., Moyer, M. P., Riecken, E. O., Buhr, H. J. and Hanski, C. (1999) Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. Proc. Natl. Acad. Sci. U.S.A **96**, 1603–1608
16 Tetsu, O. and McCormick, F. (1999) β-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature (London) **398**, 422–426
17 Crawford, H. C., Fingleton, B. M., Rudolph-Owen, L. A., Goss, K. J., Rubinfeld, B., Polakis, P. and Matrisian, L. M. (1999) The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. Oncogene **18**, 2883–2891
18 Moon, R. T. and Kimelman, D. (1998) From cortical rotation to organizer gene expression: toward a molecular explanation of axis specification in *Xenopus*. Bioessays **20**, 536–545
19 Young, C. S., Kitamura, M., Hardy, S. and Kitajewski, J. (1998) Wnt-1 induces growth, cytosolic beta-catenin, and Tcf/Lef transcriptional activation in Rat-1 fibroblasts. Mol. Cell Biol. **18**, 2474–2485
20 Ziemer, L. T., Pennica, D. and Levine, A. J. (2001) Identification of a mouse homolog of the human BTEB2 transcription factor as a β-catenin-independent Wnt-1-responsive gene. Mol. Cell Biol. **21**, 562–574
21 Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pietsch, T., Karsten, U., van de Wetering, M., Clevers, H., Schlag, P. M., Birchmeier, W. and Behrens, J. (2002) Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol. Cell Biol. **22**, 1184–1193
22 Jho, E. H., Zhang, T., Domon, C., Joo, C. K., Freund, J. N. and Costantini, F. (2002) Wnt/β-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol. Cell Biol. **22**, 1172–1183
23 Leibovitz, A., Stinson, J. C., McCombs, W. B. III, McCoy, C. E., Mazur, K. C. and Mabry, N. D. (1976) Classification of human colorectal adenocarcinoma cell lines. Cancer Res. **36**, 4562–4569
24 Brattain, M. G., Fine, W. D., Khaled, F. M., Thompson, J. and Brattain, D. E. (1981) Heterogeneity of malignant cells from a human colonic carcinoma. Cancer Res. **41**, 1751–1756
25 Moyer, M. P., Manzano, L. A., Merriman, R. L., Stauffer, J. S. and Tanzer, L. R. (1996) NCM460, a normal human colon mucosal epithelial cell line. In Vitro Cell Dev. Biol. Anim. **32**, 315–317
26 Vaidya, A. B., Lasfargues, E. Y., Sheffield, J. B. and Coutinho, W. G. (1978) Murine mammary tumor virus (MuMTV) infection of an epithelial cell line established from C57BL/6 mouse mammary glands. Virology **90**, 12–22
27 Nanni, P., de Giovanni, C., Lollini, P. L., Nicoletti, G. and Prodi, G. (1983) TS/A: a new metastasizing cell line from a BALB/c spontaneous mammary adenocarcinoma. Clin. Exp. Metastasis **1**, 373–380
28 Walter, I., Fleischmann, M., Klein, D., Muller, M., Salmons, B., Gunzburg, W. H., Renner, M. and Gelbman, W. (2000) Rapid and sensitive detection of enhanced green fluorescent protein expression in paraffin sections by confocal laser scanning microscopy. Histochem. J. **32**, 99–103
29 Hubank, M. and Schatz, D. G. (1999) cDNA representational difference analysis: a sensitive and flexible method for identification of differentially expressed genes. Methods Enzymol. **303**, 325–349
30 Block, C., Janknecht, R., Herrmann, C., Nassar, N. and Wittinghofer, A. (1996) Quantitative structure-activity analysis correlating Ras/Raf interaction *in vitro* to Raf activation *in vivo*. Nat. Struct. Biol. **3**, 244–251
31 Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. and Clevers, H. (1997) Constitutive transcriptional activation by a β-catenin-Tcf complex in APC$^{-/-}$ colon carcinoma. Science **275**, 1784–1787
32 Hazel, T. G., Nathans, D. and Lau, L. F. (1988) A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. Proc. Natl. Acad. Sci. U.S.A **85**, 8444–8448
33 Chang, C., Kokontis, J., Liao, S. S. and Chang, Y. (1989) Isolation and characterization of human TR3receptor: a member of steroid receptor superfamily. J. Steroid Biochem. **34**, 391–395
34 Song, K. H., Park, J. I., Lee, M. O., Soh, J., Lee, K. and Choi, H. S. (2001) LH induces orphan nuclear receptor Nur77 gene expression in testicular Leydig cells. Endocrinology **142**, 5116–5123
35 van Laar, T., Schouten, T., Hoogervorst, E., van Eck, M., van der Eb, A. J. and Terleth, C. (2000) The novel MMS-inducible gene Mif1/KIAA0025 is a target of the unfolded protein response pathway. FEBS Lett. **469**, 123–131
36 Nomura, N., Miyajima, N., Sazuka, T., Tanaka, A., Kawarabayasi, Y., Sato, S., Nagase, T., Seki, N., Ishikawa, K. and Tabata, S. (1994) Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1 (supplement). DNA Res. **1**, 47–56
37 Stambolic, V., Ruel, L. and Woodgett, J. R. (1996) Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. Curr. Biol. **6**, 1664–1668
38 Li, H., Kolluri, S. K., Gu, J., Dawson, M. I., Cao, X., Hobbs, P. D., Lin, B., Chen, G., Lu, J., Lin, F. et al. (2000) Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. Science **289**, 1159–1164
39 Heinen, C. D., Richardson, D., White, R. and Groden, J. (1995) Microsatellite instability in colorectal adenocarcinoma cell lines that have full-length adenomatous polyposis coli protein. Cancer Res. **55**, 4797–4799
40 Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. (1997) Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science **275**, 1787–1790
41 Perlmann, T. and Jansson, L. (1995) A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. Genes Dev. **9**, 769–782
42 Lee, H. J., Kokontis, J., Wang, K. C. and Chang, C. (1993) The use of a DNA-binding domain replacement method for the detection of a potential TR3 orphan receptor response element in the mouse mammary tumor virus long terminal repeat. Biochem. Biophys. Res. Commun. **194**, 97–103
43 Nusse, R. and Varmus, H. E. (1982) Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell **31**, 99–109

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