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ORIGINAL ARTICLE

Repressor roles for TCF-4 and Sfrp1 in Wnt signaling in breast cancer

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Mutations in Wnt pathway genes are rare in human breast cancer, yet activation of the pathway is evident from the misolocalization of β-catenin. We searched for relationships in the expression of Wnt pathway genes and found that both secreted frizzled related protein 1 (Sfrp1) and TCF-4 transcripts were all highly downregulated in a common subset of breast cancers relative to normal breast tissue. Sfrp1 has been previously characterized as a Wnt inhibitor, and we found that interfering with its expression in the human mammary epithelial cell line MCF10A activated Wnt signaling. Reduction of TCF-4 levels in breast cancer was surprising as it is a transcription factor that is responsive to Wnt signaling. Therefore, we investigated a possible inhibitory role for TCF-4 in human breast cells as well as further characterizing Sfrp1. We identified CD24 as a Wnt target in MCF10A cells and used its expression a marker of Wnt signaling. Interfering with either Sfrp1 or TCF-4 in this cell line enhanced CD24 expression. Furthermore, removal of TCF/LEF binding sites in a CD24-luciferase reporter resulted in elevated reporter gene expression. Our results indicate that both Sfrp1 and TCF-4 repress Wnt signaling in breast tissue and their downregulation contributes to the activation of Wnt signaling.
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Introduction

The oncogenic activity associated with Wnt signaling was first recognized by the ability of Wnt-1 to promote tumor formation when ectopically expressed in mouse mammary tissue (Nusse and Varmus, 1982). Paradoxically, Wnt ligands themselves have never been formally implicated in contributing to human cancers. However, two tumor suppressors in the Wnt pathway, Axin and APC, and the proto-oncogene β-catenin, are commonly mutated in a variety of human neoplasias (Polakis, 2000; Giles *et al*., 2003). Mutations in these genes deregulate the Wnt pathway by stabilizing β-catenin. Normally associated with cadherins at the plasma membrane, the uncomplexed form of β-catenin is stabilized in response to Wnt signaling and forms complexes with transcription factors of the TCF/LEF family (Nelson and Nusse, 2004). These HMG domain containing transcription factors contain high affinity binding sites for β-catenin in their extreme N-terminal regions (Brantjes *et al*., 2002) and the bound β-catenin recruits various accessories essential for gene activation (Hecht *et al*., 2000; Takemaru and Moon, 2000; Kramps *et al*., 2002). A hallmark of Wnt signaling in cancer is the stabilization and/or mislocalization of β-catenin, which presumably exerts its oncogenic effect by exacerbating the transcription of growth controlling genes.

Although mutations in Axin, APC and β-catenin are associated with certain types of human cancers, such as hepatocellular and colon cancers (Polakis, 2000), human breast tumors typically do not harbor mutations in any of these three genes, yet there is evidence for active Wnt signaling in this disease (Hatsell *et al*., 2003, Cowin *et al*., 2005). Aberrant nuclear and cytoplasmic localization of β-catenin in human breast cancer specimens has been observed (Bukholm *et al*., 2000; Lin *et al*., 2000; Ryo *et al*., 2001; Chung *et al*., 2004) and a recent study has documented cell autonomous signaling by Wnt ligands in certain human breast cancer cell lines (Bafico *et al*., 2004). Moreover, activation of the Wnt pathway promotes mammary tumorigenesis in several genetic mouse models of cancer (Moser *et al*., 1993; Roose *et al*., 1999; Imbert *et al*., 2001; Michaelson and Leder, 2001).

It is possible that Wnt signaling could be activated by as yet unidentified genetic defects. The Wnt pathway genes Frizzled, Dishevelled and GSK3, could, if mutated, contribute to deregulation of signaling but mutations in these genes have not, thus far, been associated with cancers (Ugolini *et al*., 1999). Signaling pathways can also be deregulated by epigenetic silencing of gene regulatory regions, which has been demonstrated for a number of human tumor suppressor genes (Feinberg and Tycko, 2004). Indeed, silencing of the Wnt inhibitor secreted frizzled related protein 1 (Sfrp1) in colorectal cancers was recently reported (Suzuki *et al*., 2002). Finally, aberrant Wnt signaling could result from excessive or unscheduled ligand-receptor pair interactions, as has been proposed for the Hedgehog pathway in assorted human malignancies (Thayer *et al*., 2003; Watkins *et al*., 2003). In this study, we have examined

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human breast cancers for any evidence of alterations in Wnt signaling and characterized two Wnt signaling components – Sfrp1 and TCF-4 – that are downregulated in a subset of human breast tumors.

Results

We initiated our analysis by examining gene expression patterns of Wnt pathway components in a large database containing human gene expression data for thousands of normal and diseased tissue specimens. Of particular note was the expression pattern of the Wnt inhibitor Sfrp1 in human breast tissue. Expression of Sfrp1 in 82 mRNA samples isolated from normal breast tissue (24 from patients without breast cancer and 58 from nondiseased tissue of patients with breast cancer) was compared to a set of 188 mRNA samples isolated from infiltrating ductal carcinomas (34 HER2-positive and 154 HER2-negative samples; further subtype information was not available). The relative amount of Sfrp1 mRNA was markedly diminished in breast tumor samples as compared to normal breast tissue in a large set of samples from different patients (Figure 1a). This loss of expression of Sfrp1 in breast cancer, which we confirmed independently by real-time polymerase chain reaction (PCR) analysis on a small collection of unmatched normal and tumor breast samples from several patients (Figure 1b) is consistent with *in situ* hybridization and Northern blot data from previous studies (Ugolini *et al*., 1999, 2001; Wong *et al*., 2002).

Secreted frizzled related protein 1 binds directly to Wnts and competes with their ability to activate the frizzled receptors (Finch *et al*., 1997). Therefore, the loss of Sfrp1 expression could contribute to enhanced Wnt signaling by further enabling presumptive Wnts produced by breast cells. To determine whether reduction in Sfrp1 could activate Wnt signaling in breast cells, we used siRNA to knockdown Sfrp1 in the immortalized, nontransformed human mammary epithelial cell line MCF10A (Soule *et al*., 1990). Endogenous levels of Sfrp1 protein were substantially reduced upon transfection with the appropriate siRNA duplexes (Figure 1c). To assess the effect of Sfrp1 knockdown on Wnt signaling, MCF10A cells were transfected with Sfrp1 siRNA or a lamin siRNA control along with TOPFLASH, a TCF/β-catenin luciferase reporter gene, or FOPFLASH, the reporter gene with mutated TCF binding sites (van de Wetering *et al*., 1997). Relative to lamin A/C siRNA control, Sfrp1 siRNA caused a modest but reproducible increase in luciferase activity that was most pronounced in the presence of Wnt-3A conditioned media (Figure 1d). Similar observations were made for the effects of Sfrp1 siRNA and Wnt-3A conditioned media treatment on the expression of endogenous cyclin D1, a known Wnt target (Tetsu and McCormick, 1999) (Figure 1e). Taken together, these results are consistent with Sfrp1 being a secreted inhibitor of Wnt signaling in human breast cells, loss of which might contribute to derepression of signaling in the presence of Wnt ligand.

Genes that share a common pattern of expression across a large tissue sample set might be coordinately regulated or bear relationships to each other with respect to function. We have developed a search tool referred to as BLIST (Basic Local Identification of Similar Transcription) that identifies mRNA transcripts exhibiting a tissue distribution pattern similar to that of a given transcript of interest. We employed BLIST to analyse a set of 82 normal and 313 cancerous (188 infiltrating ductal carcinomas, 28 lobular carcinomas and 10 infiltrating ductal carcinomas with features of lobular carcinomas) human breast tissues for transcripts whose relative abundance correlated highly with that of Sfrp1. Of the approximately 18 000 unique transcripts analysed on oligonucleotide arrays, TCF-4 was one of several genes whose expression pattern correlated highly with that of Sfrp1 with a Pearson coefficient of 0.76 (Figure 2a) (with the exception of IRX1, which was not examined in this study, the other genes with as high of a correlation to Sfrp1 expression as TCF-4 are all uncharacterized and were also not examined in any detail). Like Sfrp1, TCF-4 exhibited significant downregulation in breast tumors relative to normal breast tissue (Figure 1a). For comparison, keratin 18, an epithelial cell-specific marker, does not appear to be downregulated in the same tumor samples (Figure 1a). We performed immunohistochemical analysis of TCF-4 on breast tumor specimens to confirm downregulation of TCF-4 at the protein level. Of ten tumor specimens examined, six exhibited a dramatic reduction in TCF-4 protein in the cancer cells relative to normal ductal epithelial cells. Strong nuclear staining was observed for TCF-4 in epithelium of normal ducts that are surrounded by disorganized cancer cells exhibiting weak nuclear expression of TCF-4 (Figure 2b).

Following Wnt stimulation, β-catenin can activate transcription by interacting with TCF-1, LEF-1, TCF-3 and TCF-4 (Roose and Clevers, 1999). Therefore, it was surprising to find that one of these transcription factors is downregulated in breast cancer.

The expression patterns of all four human LEF/TCF transcription factors in a small collection of unmatched normal breast and tumor samples from different patients were analysed by real-time PCR. Although transcripts for every member of this family are easily detectable in human breast samples, only TCF-4 is appreciably downregulated in tumor samples as compared to normal tissue (Figure 3a). This expression pattern for TCF-4 is consistent with that which was observed using the information from the DNA microarray database and is consistent with TCF-4 having a role as a transcriptional repressor in this tissue. This is not unprecedented for this family of transcription factors, as TCF proteins associate with groucho/TLE family transcriptional repressors that inhibit the basal activity of target genes in the absence of Wnt signaling (Cavallo *et al*., 1998). Furthermore, the ability of TCF proteins to act as transcriptional repressors has been demonstrated genetically in a variety of organisms,

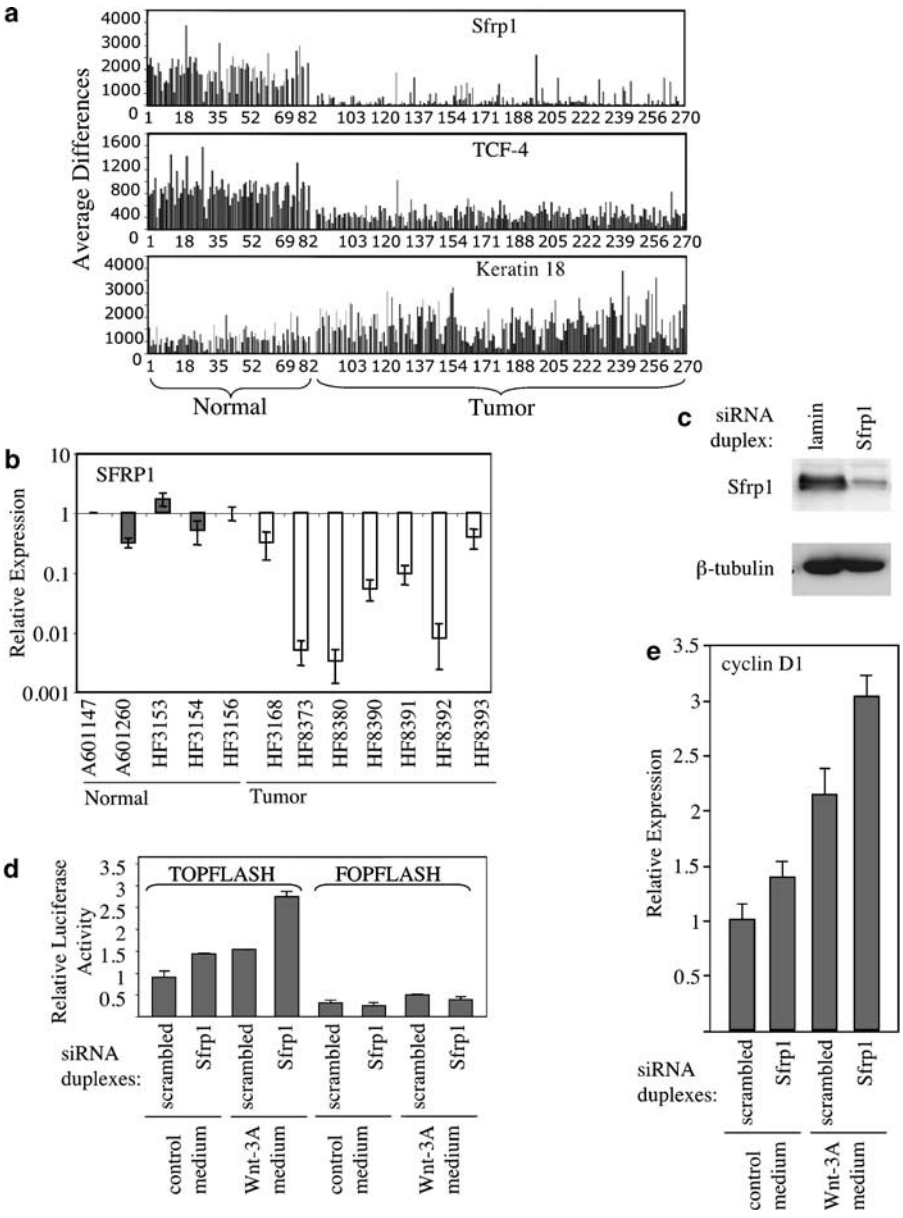

Figure 1 Reduced expression of secreted frizzled related protein 1 (Sfrp1) in human breast tumors and interference of Sfrp1 expression in cultured human mammary epithelial cells. (**a**) Oligonucleotide microarray data for Sfrp1, TCF-4 and Keratin 18 expression in 82 normal breast and 188 breast tumor samples. (**b**) Relative levels of Sfrp1 mRNA as determined by real-time polymerase chain reaction (PCR) analysis in normal breast and tumor samples. Values shown are means $\pm$ s.d. ($n=2$) from a single representative experiment. (**c**) Immunoblot of lysates from MCF10A cells transiently transfected with siRNA duplexes targeting Sfrp1 and lamin A/C using antibodies that detect Sfrp1 and β-tubulin. (**d**) Relative luciferase activity in MCF10A cells transfected with TOPFLASH or FOPFLASH along with siRNA duplexes targeting either lamin A/C or Sfrp1 and treated overnight with Wnt-3A conditioned medium or control medium starting 24 h post-transfection. Values are means $\pm$ s.d. for two independent experiments. (**e**) Relative levels of cyclin D1 levels as determined by real-time PCR in MCF10A cells transfected with either a scrambled siRNA duplex or a duplex targeting Sfrp1 and treated overnight with either Wnt-3A conditioned medium or control medium starting 24 h post-transfection. Values shown are means $\pm$ s.d. ($n=4$) from a single representative experiment.

including Xenopus, Drosophila and mice (Cavallo *et al*., 1998; Brannon *et al*., 1999; Merrill *et al*., 2004).

Both the TCF-1 and LEF1 genes possess a second promoter that drives expression of transcripts encoding forms of these proteins that lack N-terminal β-catenin binding sequence and act as transcriptional repressors (van de Wetering *et al*., 1996; Hovanes *et al*., 2001). Although a version TCF-4 that lacks the ability to bind β-catenin has not yet been identified, the TCF-4 gene has a putative transcriptional site within its second intron that would produce a protein lacking the β-catenin binding domain (Duval *et al*., 2000), raising the possibility that in breast cells, TCF-4 may act as a transcriptional repressor simply on account that it may

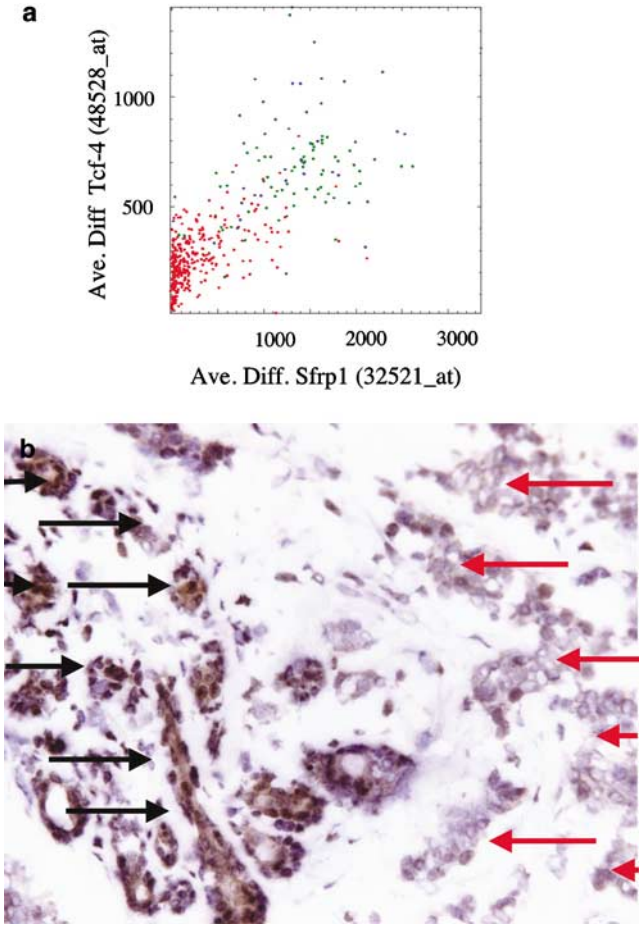

Figure 2 Reduced expression of TCF-4 in human breast cancer. (**a**) Correlations between mRNA transcript levels for secreted frizzled related protein 1 (Sfrp1) and TCF-4 in human breast tissue specimens as obtained by BLIST analysis. Each element represents an independent tissue sample where red is cancer, green is normal and blue is diseased other than cancer. Pearson correlation coefficient (r) is 0.762. (**b**) Immunohistochemical analysis of TCF-4 expression in breast tumor tissue using the anti-TCF-4 mAb 6H-5. Normal (black arrows) and cancerous (redarrows) epithelial cells are indicated. Magnification, $\times 400$.

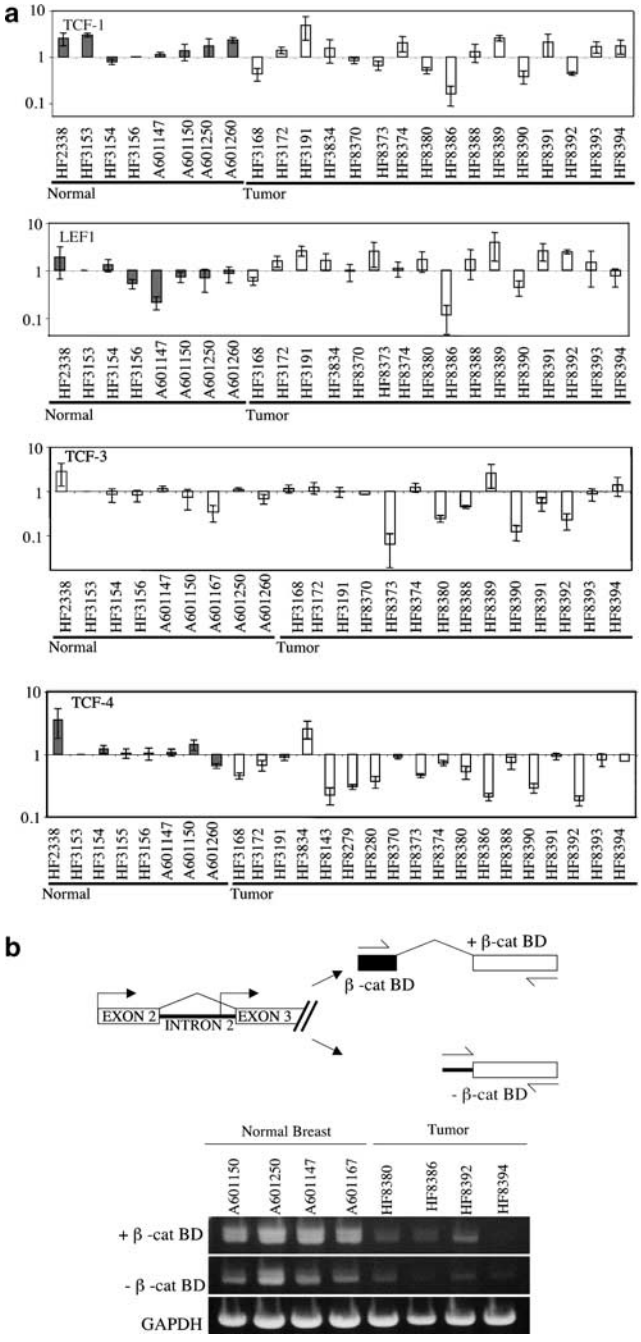

Figure 3 Expression of LEF/TCF family members in breast tissue. (**a**) Relative levels of TCF-1, LEF1, TCF-3 and TCF-4 in normal human breast samples (grey bars) and tumor samples (white bars) determined using real-time polymerase chain reaction (PCR) analysis. Values shown are means $\pm$ s.d.; $n=4$. (**b**) RT-PCR analysis of TCF-4 transcripts using primers specific to transcripts either containing or lacking the exons that encode the β-catenin binding domain ($\pm\beta$-cat BD). Shown is the putative transcriptional start site in the second intron as well as the locations of the primers used in this study. GAPDH expression was used as a control.

be unable to bind β-catenin. To test this possibility, 5′-rapid amplification of cDNA ends (5′-RACE) was used to characterize TCF-4 transcripts from MCF-7 cells. Two types of transcripts were identified: one that contains the first exon and is predicted to encode protein containing the β-catenin binding domain, and one that lacks exons 1 and 2 – presumably produced by transcription initiating within the second intron site – and would be predicted to encode the form of TCF-4 that lacks the β-catenin binding domain. Primer sets were then designed to distinguish between these two types of transcripts by RT-PCR and used to analyse human breast tissue samples (Figure 3b). Both types of transcripts were found to be present in normal breast tissue and that the transcript encoding full-length TCF-4 was at least equal in abundance to that encoding the N-terminally truncated version (Figure 3b). Moreover, both types of transcripts were downregulated in tumor tissue relative to normal. Thus, there appears to be selection against TCF-4 in breast cancer independent of its ability to bind β-catenin.

To test whether TCF-4 may act as a transcriptional repressor, we used oligonucleotide microarray analysis

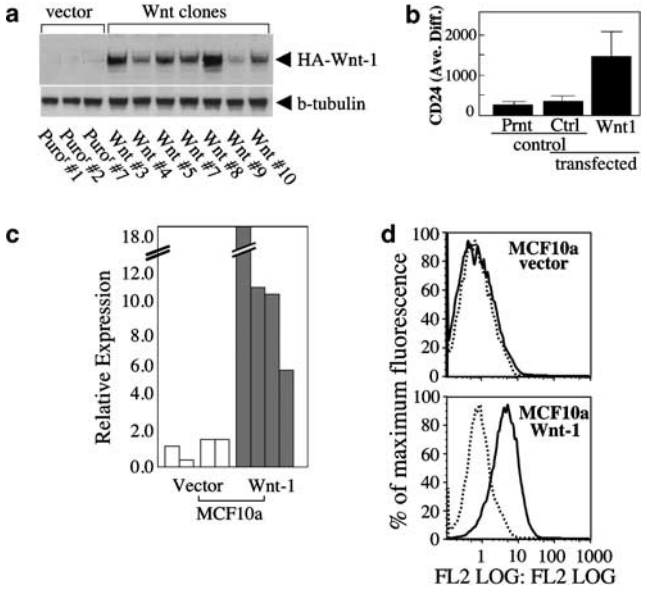

Figure 4 Overexpression of CD24 on MCF10A cells transfected with Wnt-1. (**a**) MCF10A stably expressing HA-Wnt-1 or vector alone controls were immunoblotted with anti-HA antibodies to detect HA-Wnt-1 (top) and anti-β-tubulin antibodies (bottom). (**b**) Average fluorescent signal for a CD24 probe from the oligonucleotide microarray analysis of MCF10A parental controls (Prnt), vector controls (Ctrl) and clones stably expressing Wnt-1 (Wnt). (**c**) Real-time polymerase chain reaction (PCR) analysis of CD24 expression in MCF10A vector controls and Wnt-1 stable clones. Expression of CD24 in each sample was first normalized to GAPDH expression. (**d**) Fluorescent assisted cell sorting (FACS) analysis of CD24 expression in MCF10A stably expressing Wnt-1 compared to vector alone control.

on MCF10A cells expressing Wnt-1 to identify an endogenous transcriptional marker for Wnt signaling. Constitutive expression of Wnt-1 in MCF10a leads to the stabilization of β-catenin (data not shown), which should associate with the LEF/TCF transcription factors and activate the transcription of Wnt target genes. We analysed mRNA from three independent populations of the parental MCF10A, three clones of vector control cells, and seven clones of MCF10A cells stably expressing Wnt-1 (Figure 4a). Among those genes identified by microarray analysis, CD24 mRNA was consistently detected as a transcript upregulated in Wnt-1 expressing MCF10A cells (Figures 4b and c). As CD24 mRNA codes for a cell surface sialoglycoprotein accessible to antibodies (Pirruccello and LeBien, 1986), it was possible to conduct analysis of the MCF10A clones by fluorescent activated cell sorting (FACS), revealing the presence of CD24 on cells expressing Wnt-1 but not on vector controls (Figure 4d).

Identification of CD24 as a transcriptional readout for Wnt signaling in MCF10A cells allowed us to test the effects of knocking down the transcript levels of Sfrp1, TCF-4 and LEF1 using siRNA duplexes specific to these genes. On average, transfection of each of the siRNA duplexes into MCF10A cells reduced the level of the respective endogenous transcript by at least twofold (Figure 5a). An approximate threefold increase in CD24 mRNA was observed upon treatment of MCF10A cells with siRNA specific to Sfrp1, and a twofold increase was consistently observed with TCF-4 siRNA (Figure 5b). In contrast, interference with LEF1 expression did not induce the expression of CD24 in these cells. In MCF10A clones expressing Wnt-1, the elevated levels of CD24 were further increased upon treatment with Sfrp1 siRNA, whereas a similar effect was not seen with TCF-4 siRNA (Figure 5b). These results are consistent with a role for TCF-4 as a transcriptional repressor of Wnt signaling in breast cells. Although we used an siRNA duplex directed against exon 1 of TCF-4 that would target only transcripts encoding full-length TCF-4 and not affect those encoding protein lacking the N-terminus, we observed similar results with an siRNA that would target both types of TCF-4 mRNAs (data not shown).

To further explore a potential repressor role for TCF-4, we cloned and sequenced a putative regulatory region of the CD24 gene containing a 1.2 kb segment of genomic DNA positioned immediately 5′ to the initiator ATG. Two sites that match the consensus sequence for TCF/LEF binding were identified within this region at positions -446 and -840 relative to the start ATG (Figure 5c). This 1.2-kb region was fused to luciferase to generate a CD24-luciferase reporter. When this reporter was cotransfected into HEK293 cells with a plasmid expressing activated β-catenin, an increase in luciferase activity was detected in comparison to cotransfection with an empty vector (Figure 5d). When fused in the opposite orientation to the luciferase reporter gene, the 1.2-kb regulatory region did not support luciferase expression activity nor respond to activated β-catenin (Figure 5d). Thus, the CD24 gene appears to be directly responsive to Wnt signaling and is a reliable marker for Wnt signaling in these cells.

To assess the role of TCF-4 binding to the CD24 regulatory region, the putative TCF/LEF sites at position -446 and -840 were deleted, either individually or together, and cotransfected either with a plasmid expressing activated β-catenin or an empty vector. Deletion of the -446 site resulted in an approximately twofold increase of luciferase activity both in the presence and absence of activated β-catenin relative to the wild-type reporter under the same conditions (Figure 5d). Deletion of the -840 site only caused a modest increase in luciferase activity both in the presence and absence of activated β-catenin (Figure 5d). When both sites were deleted, luciferase activity increased about threefold higher relative to wild-type reporter, whereas activated β-catenin did not significantly increase activity beyond that observed with the wild-type regulatory sequence (Figure 5d). When these same reporters were transfected into MCF10A cells, those lacking the -446 site, or both sites together, expressed luciferase activity approximately twofold higher than that of the wild-type reporter (Figure 5e). In contrast, the reporter lacking only the -840 site did not result in an increase in luciferase activity (Figure 5e).

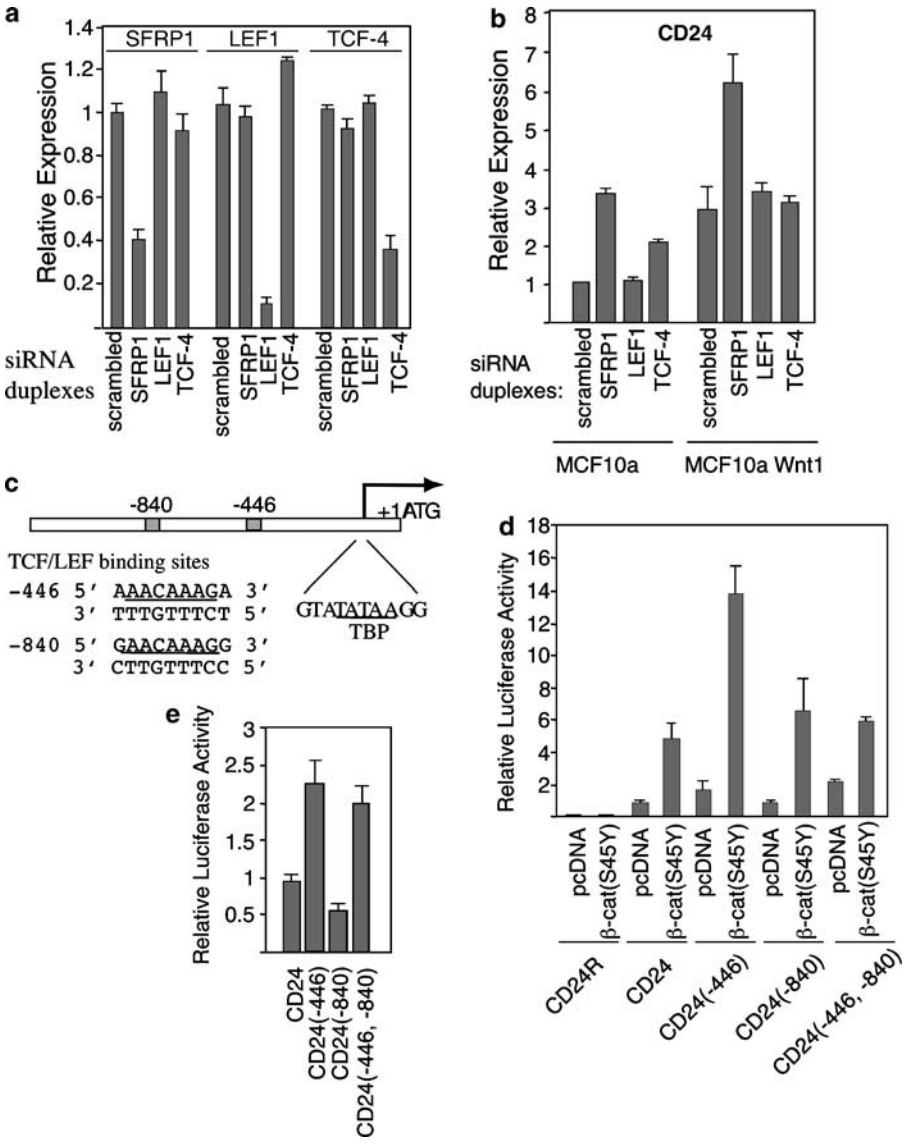

Figure 5 Regulation of CD24 expression by secreted frizzled related protein 1 (Sfrp1) and TCF-4. (**a**) Relative transcript levels as determined by real-time polymerase chain reaction (PCR) in MCF10A transfected with indicated siRNAs. Values shown are means ± s.d. ($n = 2$) of a single representative experiment. (**b**) Relative CD24 transcript levels following transfection of siRNAs specific to Sfrp1, TCF-4 or LEF1 into parental or Wnt-1 expressing MCF10A cells. Values shown are means ± s.d. ($n = 4$) of a single representative experiment. (**c**) 1.2-kb CD24 promoter showing the putative TCF/LEF binding sites. (**d**) Relative luciferase activity of HEK293 cells transfected with CD24-luciferase reporter plasmids along with either empty vector or a plasmid expressing the S45Y variant of β-catenin (β-cat S45Y). The reporter plasmids used are as follows: CD24 is the wild-type 1.2-kb CD24 promoter driving luciferase; CD24(−446), CD24(−840) and CD24(−446, −840) contain the same 1.2-kb promoter with the TCF/LEF sites at −446, the site at –840, or both sites, deleted; CD24R contains the CD24 promoter inserted in the opposite orientation. Values shown are the means ± s.d. ($n = 2$) of a single representative experiment. (**e**) Relative luciferase activity of MCF10A cells transfected with the CD24 reporter plasmids. Values shown are the means ± s.d. ($n = 2$) of a single representative experiments.

Discussion

Although genetic defects in the Wnt pathway are rare in human breast cancer there have been numerous hints that signaling is active in this disease (Hatsell *et al*., 2003). Perhaps the best evidence to date pertains to the accumulation and localization of β-catenin in human breast cancer specimens (Lin *et al*., 2000; Ryo *et al*., 2001, Chung *et al*., 2004). Cytoplasmic and nuclear β-catenin staining was observed in breast cancers in two of these studies (Lin *et al*., 2000; Ryo *et al*., 2001), whereas aberrant cytoplasmic, but not nuclear, staining was seen in others (Bukholm *et al*., 2000; Chung *et al*., 2004). The paucity of mutations in APC, Axin or β-catenin in breast cancers suggests that epigenetic mechanisms might be responsible for mislocalization of β-catenin. Such mechanisms could include increased expression of ligands, which has been reported for

various Wnts in breast cancers (Hatsell *et al*., 2003). However, Wnt ligands could remain constant yet drive excessive signaling in the absence of inhibitors that counter their activity. This could occur following reduced expression of soluble Wnt inhibitors like Sfrp1. Consistent with previous reports on smaller sample sets (Ugolini *et al*., 2001; Wong *et al*., 2002), we observed a strong reduction in Sfrp1 mRNA associated with human breast cancers.

Secreted frizzled related protein 1 has been reported to be silenced by promoter methylation in colorectal cancer (Suzuki *et al*., 2002), and we have found hypermethylation of the Sfrp1 promoter in breast tumors (data not shown), suggesting that this mechanism may also affect Sfrp1 expression in breast cancer. Reducing Sfrp1 expression by this modification could favor increased Wnt signaling by presumptive Wnts produced at steady-state levels in normal breast tissue. This argument is supported by our observations with MCF10A breast cells. Interference with Sfrp1 in these cells activated both an exogenous Wnt transcriptional reporter and two endogenous Wnt signaling targets cyclin D1 and CD24. The effects were most pronounced when Wnt3a was applied as a stimulant and demonstrate that loss of Sfrp1 enables an exogenous Wnt ligand to activate signaling. We propose that Wnt ligands are antagonized by Sfrp1 expression in normal breast, but are active in cancer when Sfrp1 is silenced.

The loss of Sfrp1 expression was apparent for the majority of the breast tumor samples represented in the oligonucleotide array database. A search for genes that shared a common pattern of downregulation in these samples revealed reduction of expression for the transcription factor TCF-4 in breast cancers. Interfering with Sfrp1 expression did not affect TCF-4 expression in MCF10A (data not shown), suggesting that reduction of TCF-4 expression is not a cause of loss of Sfrp1 expression and/or activation of the Wnt pathway.

Although it seems contradictory that downregulation of members of the family of transcription factors that are required for Wnt-induced transcription lead to aberrant propagation of the Wnt signal, there are several examples for TCF proteins acting as transcriptional repressors. During induction of endoderm in *Caenorhabditis elegans*, Wnt signaling downregulates the TCF homolog POP-1 by promoting its exclusion from the nucleus (Lo *et al*., 2004). In zebra fish, the TCF-3 homolog *hdl* represses genes driving posterior neuroectodermal fates and mutant *hdl* animals exhibit phenotypes consistent with ectopic Wnt signaling (Kim *et al*., 2000; Dorsky *et al*., 2003; Thorpe and Moon, 2004). In murine skin, TCF-3 represses epidermal differentiation independent of β-catenin binding, whereas LEF1 requires active Wnt signaling in driving features of hair differentiation (Merrill *et al*., 2001). Also, TCF-1 null mice develop intestinal and mammary tumors indicative of tumor suppressive activity for this TCF protein (Roose *et al*., 1999). Therefore, a possible role for TCF family members as tumor suppressors is not without precedent.

Gene suppression by TCF proteins could be attributed to splice variants incapable of binding β-catenin that behave in a dominant-negative fashion with respect to Wnt signaling (Roose *et al*., 1999). However, this was not the case for TCF-3 in skin as transgenes coding for full-length or N-terminal deletion mutants behaved similarly in repressing epidermal differentiation (Merrill *et al*., 2001). In this study, we observed the presence of both full-length and N-terminally deleted version of TCF-4 in breast cells. Furthermore, a TCF-4 siRNA duplex that recognizes a sequence in exon 1 and only targets transcripts encoding full-length TCF-4 resulted in increased CD24 expression. Both of these findings support a role of full-length TCF-4 as a transcriptional repressor in specific contexts.

We identified CD24 as a transcriptional target of Wnt signaling in a nontransformed human mammary epithelial cell line MCF10A. Although CD24 has been reported to be a marker for human breast cancers (Kristiansen *et al*., 2003) we did not observe any increased expression in human breast tumors that we analysed (data not shown) and we are not purporting a functionally significant role it in neoplastic transformation by Wnts. In this study, we simply used CD24 as an endogenous marker for Wnt signaling in MCF10A cells in order to study the role of TCF-4 as a transcriptional repressor. Interfering with TCF-4 expression by siRNA led to an increase in CD24 transcription, supporting the idea that TCF-4 represses CD24 expression in the absence of Wnt. Furthermore, basal activity of the CD24-luciferase reporter was enhanced when the TCF/LEF binding sites were deleted, suggesting the TCF-4 binding to this regulatory region represses transcription of CD24. Deletion of the TCF/LEF site at position –446 had a more pronounced affect on gene activation than deletion of the –840 site, suggesting it plays a more dominant role in repression. Since a reporter lacking both sites still responded to activated β-catenin, albeit more weakly than the wild-type promoter, it is possible β-catenin may stimulate transcription of CD24 in the absence of TCF binding. It is not clear how this occurs in the case of CD24, but β-catenin can induce transcription by associating with proteins other than those of the TCF/LEF family, including the estrogen receptor (Kouzmenko *et al*., 2004) and SOX-17 (Sinner *et al*., 2004).

Interfering with TCF-4 expression by siRNA led to an increase in CD24 transcription, supporting the idea that TCF-4 represses CD24 expression in the absence of Wnt. Treating MCF10A constitutively expressing Wnt-1 with TCF-4 siRNA did not increase CD24 expression, suggesting that in the presence of a Wnt signal, TCF-4 no longer acts as a repressor and is most likely converted to a transcriptional activator when it binds to stabilized β-catenin. Interfering with either LEF1 or TCF-4 in MCF10A expressing Wnt-1 also did not affect CD24 expression, suggesting that one or more of the other members of this family may be able to compensate for loss of either of these proteins. Indeed, we have found that MCF10A cells also express TCF-1 (data not shown).

In the case of breast cancer, it is not clear whether reduction of TCF-4 is sufficient to contribute to tumor progression, or whether a Wnt signal, possibly caused by loss of Sfrp1, is still required. If a Wnt signal is required for tumorigenesis, a reduction in the amount of TCF-4 may reduce the overall response to this signal since TCF-4 participates in the Wnt-dependent gene transcription by its association with β-catenin. However, all of the other LEF/TCF family members are present in both normal and tumor breast tissue suggesting that at least one of these proteins may compensate for the decrease in TCF-4. One possibility is that LEF1 may serve this function. Interfering with LEF1 by siRNA in MCF10A did not affect CD24 transcription nor does LEF1 expression appear to be reduced in breast tumors suggesting that LEF1 may not be a transcriptional repressor-like TCF-4. Therefore, not only would derepression of transcription in cells with reduced TCF-4 be unaffected by the presence of LEF1, but LEF1 would be able to associate with stabilized β-catenin in response to a Wnt signal and allow further stimulation of transcription. Although the contribution of individual Wnt signaling components to breast cancer has yet to be elucidated, it is apparent that the expression of specific components of this pathway is dramatically altered in some breast cancers and our results suggest that derepression of signaling is an outcome of these alterations.

Materials and methods

Cell culture
MCF-7 and HEK293 cell lines were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 supplemented with 10% fetal bovine serum. MCF10A cells were grown in DMEM:Ham's F-12 (1:1) supplemented with 5% horse serum, EGF, insulin, hydrocortisone, cholera toxin and antibiotics (Soule *et al*., 1990).

Anti-secreted frizzled related protein 1 antibody
Recombinant Sfrp1 was expressed and purified from *Escherichia coli* and used to immunize New Zealand rabbits to generate anti-Sfrp1 polyclonal antibodies.

Real-time polymerase chain reaction
Quantitative PCR was performed using the ABI PRISM 7700 (Applied Biosystems, Foster City, CA, USA). The Taqman® primer and probe sets used in these studies are listed in Supplementary Table 1. Relative expression of each of the genes being analysed was normalized to either GAPDH or RPL19 to account for variability in the quantity and quality of the RNA samples.

Oilgonucleotide array analysis
For the analysis of MCF10A cells or clonal derivatives, thereof, approximately 10 μg of total RNA from each cell line served as starting material for the preparation of probes required for oligonucleotide array analysis on the Affymetrix® GeneChip U133A. Probes were prepared according to previously described protocols (Wodicka *et al*., 1997). Hybridization and data analysis were performed following the protocol according to the manufacturer. For the analysis of Sfrp1mRNA expression in multiple human normal and tumor breast samples (Figures 1a and 2a), the Affymetrix data were obtained from Gene Logic Inc. (Gaithersburg, MD, USA). In the data presented in these figures, there are a total of 270 samples: 82 normal breast and 188 tumor. GeneLogic data were also normalized using global scaling but in this instance the target intensity was 100. The Affymetrix data for Sfrp1 were generated from the U95 probe set ID 32521_at, the data for TCF-4 were generated from the U95 probe set ID 48528_at and the data for Keratin 18 were generated from the U95 probe set ID 35766_at.

To identify genes whose tissue expression pattern correlates positively with that of a given gene of interest, a query tool referred to as BLIST was devised. This tool computes the similarity of the gene expression profile of a given probe set to that of all other probe sets in the GeneLogic database of micro array data. Similarity between gene expression profiles is measured as the Pearson coefficient between the expression values for the two probe sets over the 395 breast tissue specimens in the database. The query returns probe sets ranked in descending order of Pearson correlation coefficient.

5′-rapid amplification of cDNA ends and real-time-polymerase chain reaction analysis
5′-rapid amplification of cDNA ends was performed using the BD SMART™ RACE cDNA Amplification Kit (BD Biosciences, San Jose, CA, USA). Total RNA isolated from MCF-7 was used as the template for the reverse transcriptase reaction, and the resulting cDNA was used in PCR using a primer specific for TCF-4 oriented towards the 5′ end of the transcript (see Supplementary Table 2). The Qiagen® One-Step RT-PCR Kit (Qiagen) was used for RT-PCR analysis of TCF-4 using one primer set specific for transcripts containing the first two exons and one primer set specific for transcripts lacking these two exons but containing the second intron (see Supplementary Table 2).

Immunohistochemistry
Breast tissue cryo-sections were fixed with 100% acetone and stained using the Vectastain™Elite ABC glucose oxidase system (Vector Labs, Burlingame, CA, USA) and the Metal Enhanced DAB Substrate Kit (Pierce, Rockford, IL, USA). Anti-TCF-4 6H-5 mAb (Upstate, Charlottesville, VA, USA) was used at a final concentration of 10 μg/ml and detected using a 1:200 dilution of the biotinylated anti-mouse antibody. Nuclei were detected by counterstaining with Mayer's hematoxylin.

siRNA knockdown of GAPDH, secreted frizzled related protein 1 and TCF-4 in MCF10A
MCF10A was transfected with siRNA duplexes directed against each target as described in the text (see Supplementary Table 2 for siRNA sequences) using Oligofectamine™(Invitrogen, Carlsbad, CA, USA) transfection reagent according to the previous publication (Reginato *et al*., 2003). Cells were harvested at the indicated times and total RNA was prepared using RNeasy (Qiagen, Valencia, CA, USA).

Fluorescent activated cell sorting analysis
For FACS analysis, MCF10A and MCF10A stably expressing Wnt-1 were labeled with either PE-conjugated anti-human CD24 (BD Biosciences) or a PE conjugated IgG_{2a}, κ isotype control (BD Biosciences) in FACS buffer (PBS containing 1% FBS and 2 mM EDTA).

CD24-luciferase. reporter plasmids
Human genomic DNA (Promega, Madison, WI, USA; catalog number G304A) was used as a template for PCR to isolate a 1.2-kb region of DNA extending from position −1167 to position −30 relative to the CD24 start codon (where the A of the start ATG is +1). This 1.2-kb region was then inserted into pGL3-Basic (Promega) to generate the CD24 luciferase reporter plasmids described in this study. Polymerase chain reaction was used to generate complete deletions of the putative TCF/LEF binding sites at positions −446 and −840.

Luciferase assays
HEK293 cells were transfected in a 12-well plate at 5×10^5 cells per well using Lipofectamine 2000 (Invitrogen) transfection reagent. Transfections were carried out in triplicate using 100 ng pRL-SV40, 1 μg of the appropriate CD24 promoter luciferase reporter plasmid and 1 μg of the S45Y β-catenin construct or empty vector per transfection. MCF10A cells were transfected using the Nucleofector Device (Amaxa, Cologne, Germany) as recommended by the manufacturer. Firefly and *Renilla* luciferase activities were assayed 48 h following transfection using the dual Luciferase Assay System (Promega). Firefly luciferase was normalized to *Renilla* luciferase activity to correct for variability in transfection efficiencies.

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Supplementary Information accompanies the paper on Oncogene website (http://www.nature.com/onc).