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High-Frequency Canonical Wnt Activation in Multiple Sarcoma Subtypes Drives Proliferation through a TCF/β-Catenin Target Gene, *CDC25A*

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SUMMARY

Wnt canonical signaling is critical for normal development as well as homeostasis of several epithelial tissues, and constitutive activation of this pathway is commonly observed in carcinomas. We show here that 50% of human sarcomas (n = 45) and 65% of sarcoma cell lines (n = 23) of diverse histological subtypes exhibit upregulated autocrine canonical Wnt signaling. Furthermore, in Wnt autocrine cell lines, we identify alterations including overexpression or gene amplification of Wnt ligands and/or *LRP5/6* coreceptors and epigenetic silencing of different cell surface Wnt antagonists. Mutations in adenomatous polyposis coli (*APC*) gene were observed in two nonautocrine Wnt-positive sarcoma cell lines. Finally, downregulation of the activated Wnt pathway inhibited sarcoma cell proliferation both in vitro and in vivo by a mechanism involving the downregulation of CDC25A.

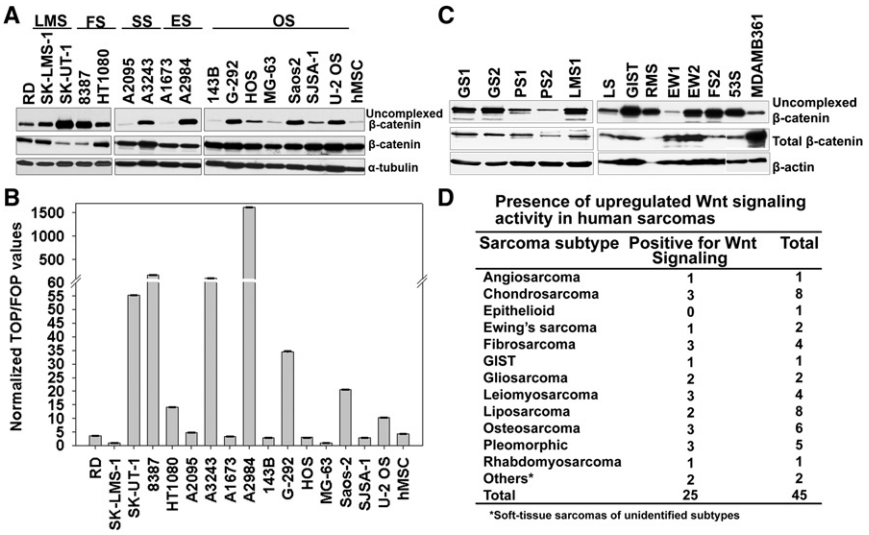
INTRODUCTION

The Wnt family of secreted proteins serves evolutionarily conserved functions in normal development and adult tissue homeostasis (Clevers, 2006). Wnt signaling through its receptors activates intracellular-signaling networks for at least three distinct pathways, including canonical Wnt/β-catenin, noncanonical planar cell polarity, and Wnt/Ca^{2+}+ pathways (MacDonald et al., 2009; van Amerongen and Nusse, 2009). The canonical Wnt signaling is initiated when a Wnt ligand binds to a cell surface receptor complex consisting of Frizzled (Fz) and low-density lipoprotein receptor-related proteins (LRPs) 5 and 6, leading to the phosphorylation of LRP5/6 coreceptors. This ligand-receptor interaction further results in the inactivation of a degradation complex, consisting of adenomatous polyposis coli (APC), axin, and glycogen synthase kinase 3 (GSK3)β, which in the absence of Wnt ligand, targets β-catenin for proteosomal degradation (Clevers, 2006). β-Catenin then accumulates in the cytoplasm, translocates to the nucleus, and binds to T cell-specific transcription factor/lymphoid-enhancer binding factor (TCF/LEF) complex to modulate TCF-dependent transcription of target genes. Certain TCF/β-catenin target genes, including *AXIN2*, *c-MYC*, and *LEF1*, appear to be transcriptionally upregulated in a tissue-independent manner, whereas other target genes may be tissue or context specific (He et al., 1997; Hovanes et al., 2001; Jho et al., 2002; Leung et al., 2002).

Wnt signaling is kept in check at multiple levels through receptor downregulation (Khan et al., 2007) and feedback-negative regulators such as AXIN2 and DKK1 (Bafico et al., 2001; Jho et al., 2002; Wu et al., 2000). DKKs bind to LRP5/6 and inhibit Wnt canonical signaling (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001; Tamai et al., 2000). Another class of antagonists, FRPs, binds to and sequesters Wnts, blocking both canonical and noncanonical signaling (Bafico et al., 1999; Rattner et al., 1997). Wnt/β-catenin signaling is involved in the maintenance of normal tissue stem/progenitors of epithelial tissues, including the gastrointestinal tract, skin, mammary gland, and lung (Radtke and Clevers, 2005; Reya and Clevers, 2005). Carcinomas that arise in these same tissues often exhibit aberrant Wnt pathway activation by mechanisms such as

Significance

Sarcomas comprise the most common malignancy of childhood and afflict adults as well. Canonical Wnt signaling influences the maintenance of mesenchymal stem cells, and our findings indicate that sarcomas commonly select for upregulation of Wnt autocrine signaling, which acts to increase their proliferation through the functions of a TCF/β-catenin target gene, *CDC25A*, a major regulator of cell cycle progression. The high frequency at which the Wnt pathway is activated in diverse human sarcomas identifies Wnt signaling as a potential target for therapies that could decrease morbidity and mortality from this disease.



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Presence of upregulated Wnt signaling activity in human sarcomas

Sarcoma subtype	Positive for Wnt Signaling	Total
Angiosarcoma	1	1
Chondrosarcoma	3	8
Epithelioid	0	1
Ewing's sarcoma	1	2
Fibrosarcoma	3	4
GIST	1	1
Gliosarcoma	2	2
Leiomyosarcoma	3	4
Liposarcoma	2	8
Osteosarcoma	3	6
Pleomorphic	3	5
Rhabdomyosarcoma	1	1
Others*	2	2
Total	25	45

*Soft-tissue sarcomas of unidentified subtypes

Figure 1. High Frequency of Upregulated Canonical Wnt Activity in Human Sarcomas and Sarcoma Lines of Multiple Histological Subtypes

(A) Total cell lysates were subjected to precipitation with a glutathione S-transferase (GST)-E cadherin fusion protein (Bafico et al., 1998) followed by immunoblot analysis with mAb directed against β-catenin. α-Tubulin was used as a loading control.

(B) TCF luciferase reporter activity in human sarcoma cells. Results are depicted as the ratio of TOP/FOP luciferase activity at 72 hr after transduction. Error bars indicate SD of mean values obtained from triplicates and are representative of two independent experiments.

(C) Protein extracts from frozen sections of primary sarcoma tissues were subjected to analysis of uncomplexed β-catenin levels, total β-catenin, and β-actin as in (A). 53S and MDAMB361 cell lysates served as positive and negative controls (Bafico et al., 2004). A representative blot is shown.

(D) Frequency of elevated uncomplexed β-catenin levels in primary sarcomas. Results reflect at least two independent analyses for each tumor specimen. LMS, leiomyosarcoma; FS, fibrosarcoma; SS, synovial sarcoma; ES or EW, Ewing's sarcoma; OS, osteosarcoma; GS, gliosarcoma; PS, pleomorphic; LS, liposarcoma; GIST, gastrointestinal stromal tumor; RD or RMS, rhabdomyosarcoma.

See also Figure S1.

mutations of *APC*, *CTNNB1*, or *AXIN* (Giles et al., 2003; Polakis, 2000) and more recently through autocrine Wnt activation (Akiri et al., 2009; Bafico et al., 2004).

Wnt signaling is also among the developmental pathways that regulate the self-renewal and differentiation of mesenchymal stem cells (MSCs) (Hartmann, 2006; Jaiswal et al., 2000; Siddappa et al., 2008; Tezuka et al., 2002). MSCs isolated from bone marrow can differentiate along osteogenic, chondrogenic, adipogenic, connective tissue, as well as myogenic lineages (Pittenger et al., 1999). Sarcomas, which account for the majority of pediatric malignancies (Jemal et al., 2008), and occur in adults as well, involve mesenchymal tissues and comprise a diverse array of histological subtypes (Skubitz and D'Adamo, 2007). MSCs have been identified as the targets of "first-hit" in Ewing's sarcoma and malignant fibrous histiocytoma (MFH) (Matushansky et al., 2007; Miyagawa et al., 2008; Tirode et al., 2007). We recently showed that human MSCs (hMSCs) exhibit low levels of endogenous Wnt signaling and that whereas the addition of exogenous Wnt to these cells promoted their self-renewal, excess Wnt inhibited hMSC differentiation along osteogenic or adipogenic lineages (Liu et al., 2009). Based on these findings, the major goal of the present study was to investigate the role of canonical Wnt pathway in human sarcomagenesis.

RESULTS

Detection of Increased Wnt Activity in a High Fraction of Human Sarcomas of Diverse Histological Types

To investigate a possible role of Wnt pathway activation in sarcomas, we screened human tumor lines representing major sarcoma subtypes for canonical Wnt activity. We identified upregulated Wnt signaling relative to that observed in hMSCs as evidenced by increased levels of uncomplexed β-catenin (Figure 1A; see Figure S1 available online) and increased TCF reporter activity (Figure 1B) in 17 of 23 tumor lines analyzed including those derived from Ewing's, fibro-, leiomyo-, lipo-, osteo-, rhabdomyo-, and synovial sarcoma (Figure S1). We next screened both soft tissue and bone sarcomas for uncomplexed β-catenin levels. Figure 1C shows a representative blot for uncomplexed β-catenin levels in different subtypes of sarcoma cell lines relative to 53S, a previously reported Wnt-activated tumor line (Bafico et al., 2004). Among a total of 45 primary sarcomas analyzed, Wnt activation was observed in 25, representing 12 different histological subtypes (Figure 1D). These results demonstrate that the Wnt canonical pathway is upregulated in a large fraction of human sarcoma cell lines and sarcomas of diverse subtypes.

Wnt Activation in Human Sarcomas Involves Multiple Mechanisms

We initially surveyed each Wnt-positive tumor line for evidence of β-catenin activating lesions, a commonly observed mechanism for Wnt activation in tumors (Polakis, 1999), but found none among 11 lines tested. To test whether constitutive Wnt pathway activation in these sarcoma lines was due to an autocrine loop, we treated sarcoma cells with either DKK1 or FRP. Many showed pathway inhibition in response to DKK1 and/or FRP (Figures 2A and 2B), supporting an autocrine mode of Wnt pathway activation in these sarcoma lines. Neither treatment inhibited the upregulated Wnt pathway in two cell lines, A2984 and SK-UT-1 (Figures 2B; Figure S1). Thus, we analyzed these lines for APC alterations by western blotting and detected truncated APC proteins in both cell lines (Figure S2D). We then searched the Sanger Institute database on somatic mutations in cell lines and found that SK-UT-1 had been identified as having heterozygous *APC* mutations, consistent with our results of immunoblot analysis. We confirmed these mutations by sequence analysis (data not shown) and identified an insertion of "G" base at

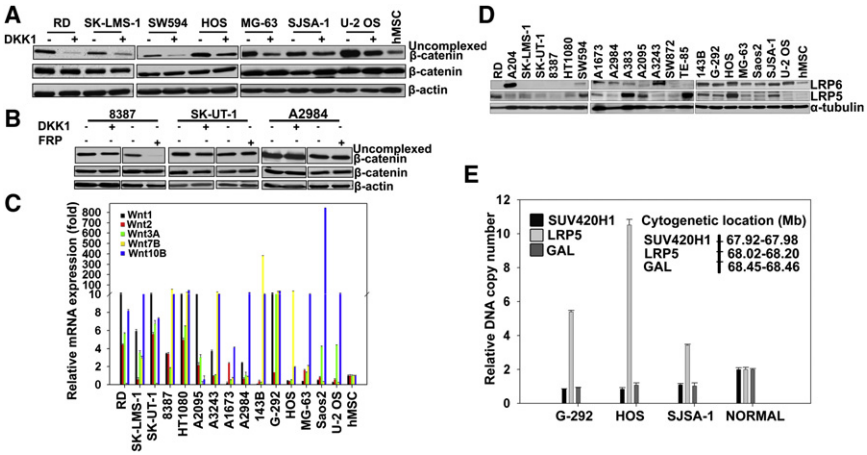

Figure 2. Wnt Autocrine Activation in Human Sarcomas Occurs Commonly and Is Associated with Several Pathway Aberrations

(A) Effects of DKK1 on Wnt/β-catenin signaling in human sarcoma cells. Cells were treated with DKK1 for 3 hr followed by analysis of uncomplexed β-catenin levels as described in Figure 1A.

(B) Wnt-activated sarcoma lines resistant to DKK1 inhibition were stably infected with human FRP followed by immunoblot analysis as described in (A).

(C) Real-time PCR analyses for various canonical Wnt ligands were conducted on total RNAs. Normalized values are represented relative to those in hMSC.

(D) Protein extracts from whole-cell lysates of different human sarcoma lines and hMSCs were subjected to immunoblot analysis.

(E) Real-time PCR was performed on genomic DNAs extracted from the indicated sarcoma lines to analyze *LRP5* gene copy number. *SUV420H1* and *GAL* are two flanking genes 5′ and 3′ of *LRP5*, respectively. Albumin was used as a standard for diploid gene copy number. Normalized values are represented relative to those in normal human epithelial cells. Error bars indicate SD of mean values obtained from triplicates and are representative of two independent experiments.
See also Figure S2.

4790_4791, resulting in an amino acid substitution of alanine to glycine and the generation of a premature stop codon in A2984 sarcoma line (Figure S2D).

To further investigate the mechanisms involved in autocrine Wnt activation, we analyzed expression levels of various canonical Wnt ligands and antagonists. The relative mRNA levels of Wnt ligands analyzed varied among sarcoma cell lines, but each line exhibited much higher levels of at least some Wnts capable of triggering canonical signaling when compared to hMSCs (Figure 2C). Additionally, some sarcoma lines showed much higher levels of LRP5 and/or LRP6 protein expression compared to hMSCs (Figure 2D). HOS and G-292 cells showed markedly increased *LRP5* mRNA (Figure S2A) and protein levels (Figure 2D), and also exhibited increased *LRP5* gene copy number (Figure 2E) when compared to flanking genes. In other sarcoma lines with increased LRP5/6 protein levels, there was no significant increase in the transcript level of either gene, implying that increased protein stabilization was responsible. In a subset of the sarcoma cell lines in our series, reduced expression of one or more Wnt antagonists correlated with the presence of promoter methylation (Figure S2B). Figure S2C summarizes relative expression levels of both positive and negative cell surface regulators of the Wnt pathway in sarcoma lines with Wnt pathway activation compared to hMSCs.

Identification of *CDC25A* as a TCF/β-Catenin Target Gene in Wnt-Activated Human Sarcoma Cells

In a microarray experiment conducted to discover potential targets of Wnt signaling in hMSCs, we identified CDC25A, an important regulator of cell cycle (Boutros et al., 2007), to be upregulated markedly in response to WNT3A treatment (Table S1) and validated this result by real-time PCR and western blotting (Figures 3A; Figure S3). As shown in Figure 3A, the relative expression levels of *CDC25A* and *AXIN2* increased by more than 4-fold within 6 hr of hMSC treatment with WNT3A. Expression levels of the other two CDC25 family members did not increase under these same conditions. Sarcoma lines of diverse subtypes with Wnt activation also showed preferential expression of *CDC25A* compared to hMSCs (Figure 3B). Moreover, analysis of a primary human sarcoma, which was positive for nuclear β-catenin by immunostaining, also stained positive for nuclear CDC25A, whereas the paired normal tissue was negative for both (Figure 3C). It should be noted that CDC25A protein expression level and the level of Wnt activation did not uniformly correlate among the sarcoma lines analyzed, which may be accounted for by the complexity of *CDC25A* regulation at posttranslational as well as transcriptional levels (reviewed in Boutros et al., 2007).

To determine whether *CDC25A* was a direct TCF/β-catenin transcriptional target, we performed an in silico analysis of the *CDC25A* promoter and identified two putative TCF-binding (TCFB) sites within a 1.3 kb fragment upstream of the first exon (Figure 3D). Chromatin immunoprecipitation (ChIP) performed on DNA extracted from Wnt-activated U-2 OS cells revealed that β-catenin associated with the two putative TCFB sites in the *CDC25A* promoter and at a similar site present within the *AXIN2* promoter (Figure 3D). When the CDC25A reporter was cotransfected with a constitutively activated S33Y β-catenin in HEK293T cells, we observed a dose-dependent increase in reporter activity (Figure 3E). Moreover, the observed CDC25A promoter activity was inhibited when the putative TCFB sites (ΔTCFB) in the CDC25A promoter were mutated (Figure 3F), further establishing *CDC25A* as a direct transcriptional target of canonical Wnt signaling.

Downregulation of Activated Wnt Signaling Inhibits Human Sarcoma Proliferation In Vitro and In Vivo

To address the biological significance of upregulated Wnt signaling in human sarcoma cells, we transduced tumor lines representing four distinct sarcoma subtypes exhibiting either autocrine Wnt activation or *APC* truncation mutations, with a constitutive dnTCF4 lentivirus. Using this strategy, we observed greater than 50% inhibition of TCF reporter activity (Figure 4A) associated with markedly decreased colony-forming ability in

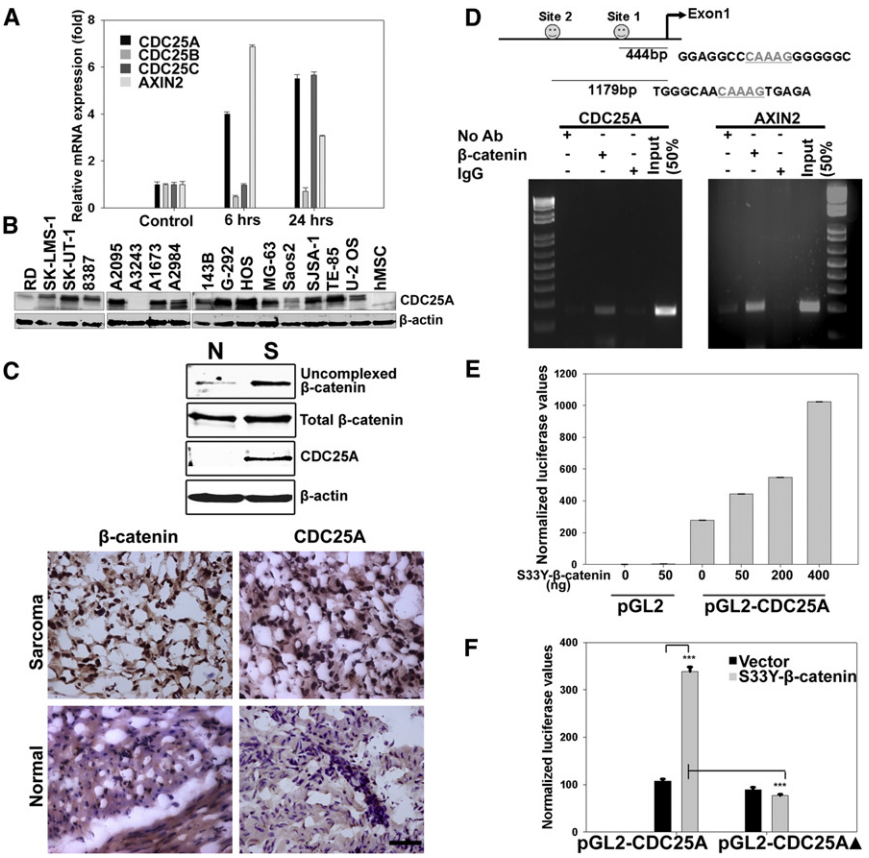

Figure 3. CDC25A Is a Direct TCF/β-Catenin Transcriptional Target

(A) hMSCs were treated with 100 ng/ml of WNT3A for either 6 or 24 hr, and total RNA was extracted. Real-time PCR was conducted to measure the mRNA expression changes for the indicated genes as described in Figure 2C.

(B) Whole-cell lysates of different human sarcoma lines and hMSCs were subjected to immunoblot analysis for expression of CDC25A.

(C) Uncomplexed β-catenin levels in a primary human leiomyosarcoma and corresponding adjacent normal tissue were determined as described in Figure 1. Protein extracts from tissues were subjected to immunoblot analysis with specific antibodies to β-catenin and CDC25A (top). Immunostaining for β-catenin or CDC25A was performed on frozen sections (bottom) from the same leiomyosarcoma and corresponding normal tissue as used for western blotting above. Scale bar indicates 50 μm.

(D) Schematic representation of *CDC25A* promoter region containing putative TCFB elements (top) and ChIP assay in autocrine Wnt-activated U-2 OS osteosarcoma cells (bottom).

(E) Empty PGL2 luciferase reporter vector or a reporter construct containing the 1.3 kb promoter region of *CDC25A* was transfected into HEK293T cells with or without an expression vector for constitutively active S33Y-β-catenin. Luciferase reporter activities were measured 48 hr posttransfection.

(F) Site-directed mutagenesis was used to mutate the two putative TCFB elements in the 1.3 kb *CDC25A* promoter region. Wild-type or mutant CDC25A reporter constructs (200 ng each) were cotransfected with a constitutively active S33Y-β-catenin expression construct (50 ng) in HEK293T cells. Luciferase activities were measured 48 hr posttransfection and normalized to Renilla as in (E). ***$p < 0.001$ by Student's t test. Error bars indicate SD of mean values from triplicates and are representative of two independent experiments.

See also Figure S3 and Table S1.

sarcoma cells having either mechanism of Wnt activation (Figure 4B). As a specificity control, dnTCF4 expression did not significantly alter colony formation by A1673, a sarcoma line with very low or undetectable levels of uncomplexed β-catenin (Figures 1A and 4B). Analyses of cell cycle profiles of sarcoma lines expressing dnTCF4 indicated that growth inhibition was achieved either via G1 or G2 arrest (Figure 4C). We also tested the effects of shRNA knockdown of either *LRP5* or *LRP6* in HOS and A204 sarcoma lines, which exhibited high levels of endogenous LRP5 and LRP6 proteins, respectively. Downregulation of these receptors led to a concomitant decrease in Wnt reporter activity and inhibition of in vitro proliferation (Figure S4A), implying the involvement of these overexpressed receptors in autocrine Wnt-mediated proliferation of these sarcoma lines. In addition, the ectopic expression of a constitutively active β-catenin rescued FRP-mediated inhibitory effects of Wnt downregulation on in vitro proliferation, further confirming the involvement of the canonical Wnt pathway (Figure S4B).

Because CDC25A plays a crucial role in both G1/S and G2/M progression (reviewed in Boutros et al., 2007), we further analyzed CDC25A levels in representative Wnt-activated sarcoma cell lines. We observed in all cases that CDC25A protein (Figure 4D) and mRNA (Figure S4C) levels were significantly reduced when Wnt signaling was inhibited by dnTCF4 as well as by DKK1, FRP, and shRNA directed against *LRP5* (Figure S4D). Each approach downregulated *CDC25A* transcript levels comparably to *AXIN2*. In contrast, there were no significant effects on *CDC25B* and *CDC25C* mRNA levels under the same conditions. Furthermore, DKK1 inhibition of CDC25A expression was detectable by 36 hr, prior to any observable alteration of cell cycle profile (Figure S4E). For comparison, we also measured transcript levels of the three *CDC25* genes in HT1080 sarcoma cells synchronized either at G1/S or G2/M phases. Expression levels of all three *CDC25* genes showed similar decreases in cells arrested in G1/S (Figure S4F), whereas Wnt pathway inhibition by dnTCF4 resulted in specific downregulation of *CDC25A* transcript levels as indicated above. All of these results argue that CDC25A downregulation observed as a result of Wnt pathway inhibition is not due to a cell cycle positional effect and confirm our conclusions that *CDC25A* is a direct Wnt transcriptional target gene.

We next compared the effects of downregulating TCF signaling or CDC25A in Wnt autocrine HT1080 cells. Constitutive dnTCF4 expression significantly inhibited tumor formation (Figures 5A and 5B), confirming the inhibition of cell proliferation observed in vitro (Figure 4B). Of note, exogenous CDC25A expression in these same cells rescued in vivo tumor growth (Figure 5A), whereas downregulation of CDC25A alone using a specific

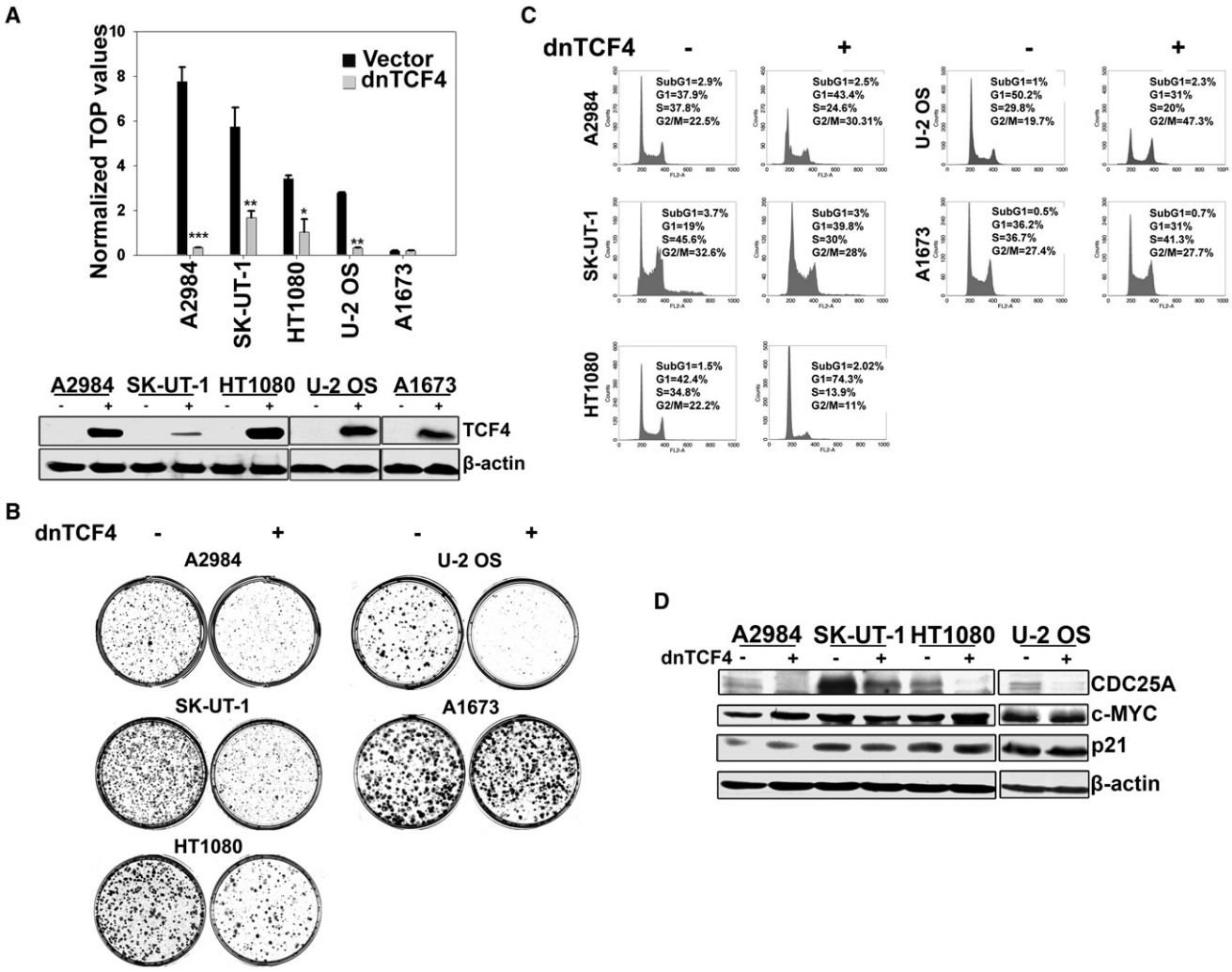

Figure 4. Downregulation of Activated Wnt Signaling in Human Sarcoma Cells Induces Inhibition of Proliferation In Vitro

(A) Downregulation of Wnt signaling in sarcoma lines constitutively expressing lentiviral-transduced dnTCF4 was assessed by TCF luciferase reporter assay as in Figure 1B. Error bars indicate SD of mean values from triplicates and are representative of two independent experiments (top). Western blot analysis was performed using whole-cell lysates from sarcoma lines expressing dnTCF4 (bottom).

(B) Human sarcoma lines were transduced with either vector control or dnTCF4-expressing lentiviruses. In vitro proliferation was assessed by colony-forming assays. Results are representative of at least two independent experiments.

(C) Cell cycle profile analysis of sarcoma cells stably expressing dnTCF4 performed by FACS.

(D) Cell lysates from sarcoma lines expressing dnTCF4 were subjected to immunoblot analysis for CDC25A, c-MYC, and p21.

See also Figure S4.

shRNA (Figures 5C and 5D) resulted in decreased HT1080 tumor formation (Figures 5A and 5B). Ectopic CDC25A also rescued dnTCF4-mediated inhibition of cell proliferation with several additional Wnt-activated sarcoma cell lines analyzed (Figure S4G). These findings establish that Wnt signaling through the TCF/β-catenin target gene, *CDC25A*, contributed significantly to tumor formation of these Wnt-activated sarcomas.

Comparative Analysis of *CDC25A* and *c-MYC* Expression Identifies *CDC25A* as a Major Mediator of β-Catenin Driven Sarcoma Cell Proliferation

c-MYC is a direct Wnt transcriptional target in colon and other carcinoma cells (Finch et al., 2009; He et al., 1998; Sansom et al., 2007; van de Wetering et al., 2002) As shown in Figure 6, dnTCF4 expression reduced c-MYC levels in HCT116, a *β-catenin* mutated colon cancer line reported to have high Wnt activity (He et al., 1998; Morin et al., 1997; Suzuki et al., 2004) (Figure 6A, lanes 1–2). In contrast, downregulation of Wnt signaling in several sarcoma lines failed to significantly affect c-MYC levels, although CDC25A levels were reduced in each case (Figure 4D and Figure 6A, lanes 3–8). The CDK inhibitor, *p21*, is a known *c-MYC* repression target and is upregulated in response to *c-MYC* downregulation in colon (van de Wetering et al., 2002) and other Wnt-activated carcinomas (Akiri et al., 2009; Bafico et al., 2004; van de Wetering et al., 2002). However, Wnt-signaling downregulation had no effect on p21 levels in the sarcoma lines analyzed (Figure 4D). Whereas WNT3A significantly induced *CDC25A* expression in hMSCs (Figure 3A), WNT3A treatment of these cells

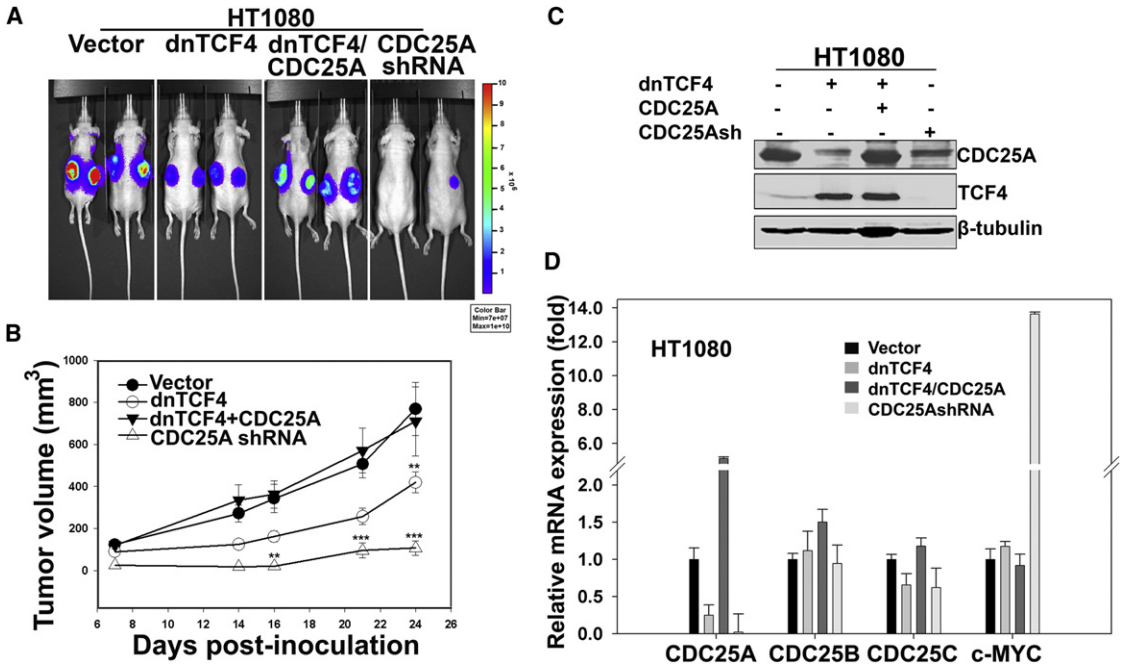

Figure 5. Downregulation of Activated Wnt Signaling in HT1080, a Human Fibrosarcoma Cell Line, Induces Inhibition of Proliferation In Vivo

(A) HT1080 cells stably expressing firefly luciferase were transduced with lentiviruses expressing the indicated genes and marker selected. Cells (1×10^6) were injected subcutaneously in nude mice (n = 4), and whole animal imaging was performed 6 weeks post-inoculation.

(B) Graph showing tumor growth over time measured by calipers. Each data point represents a mean of eight values (n = 4 mice/group), and error bars indicate SD of mean values. **p < 0.01, ***p < 0.001 by two-way ANOVA.

(C) Lysates from HT1080 cells expressing the indicated constructs prior to inoculation in mice were subjected to immunoblot analysis.

(D) Real-time PCR was conducted to measure the mRNA expression changes for the specified genes as described in Figure 2C. Error bars indicate SD of mean values from triplicates.

did not significantly alter mRNA levels of *c-MYC* or *CyclinD1* (Figure 6B), another reported Wnt target gene (Tetsu and McCormick, 1999). These results indicate that neither is a direct transcriptional target of Wnt signaling in hMSCs. All of these findings argue that *CDC25A*, but not *c-MYC*, is a TCF/β-catenin transcriptional target in hMSCs and sarcoma cells.

When we analyzed c-MYC protein levels in human sarcoma lines, strikingly, most exhibited upregulated c-MYC expression as compared to hMSCs (Figure 6C). Primary sarcomas also exhibited increased c-MYC protein levels compared to their normal tissue counterparts (Figure 6D). These results imply that a Wnt-independent mechanism involving increased protein stabilization frequently increases c-MYC protein expression in sarcomas. Moreover, both *c-MYC* and *CDC25A*-specific shRNAs inhibited in vitro proliferation of A2984 and HT1080 sarcoma as well as HCT116 carcinoma cells, implying that both genes contribute significantly to proliferation of these tumor cells (Figure S5). *CDC25A* is also known to be a *c-MYC* transcriptional target gene in mammalian cells (Galaktionov et al., 1996). To test whether CDC25A was also a direct TCF/β-catenin target in carcinoma cells, we transfected HCT116 and Wnt autocrine U-2 OS cells with the CDC25A reporter and compared relative activities of both wild and mutant reporter constructs. In both tumor lines the wild-type CDC25A reporter exhibited activity that was inhibited in the presence of dnTCF4 (Figure 6E). When U-2 OS and HCT116 cells were cotransfected with the mutant (ΔTCFB)-CDC25A reporter in the presence or absence of dnTCF4, reporter activity was further decreased in HCT116 but not in U-2 OS cells, presumably due to reduced c-MYC transcriptional function specific to the carcinoma cells (Figure 6E). Thus, it is likely that *CDC25A* is both a direct and indirect TCF/β-catenin target in Wnt-activated carcinoma cells.

Wnt Activity and In Vitro Differentiation Potential of Human Sarcoma Cell Lines

High levels of Wnt signaling inhibit differentiation of hMSCs along osteogenic or adipogenic lineages in vitro and in vivo (Liu et al., 2009). Osteosarcoma cell lines, G-292, Saos2, SJSA-1, and TE-85, exhibited the ability to differentiate along the osteogenic lineage, as detected by staining for two markers of osteogenic differentiation, alkaline phosphatase (AP) and alizarin red S (AR) (Figure 7A). Wnt signaling is downregulated in MSCs during later stages of osteogenic differentiation, allowing complete maturation of osteoblasts to osteocytes (Li et al., 2005). We observed a similar suppression of Wnt activity in G-292 and TE-85 cell lines in which Wnt signaling was downregulated by more than 80% under osteogenic induction conditions (Figures 7B and 7C). In U-2 OS, which failed to differentiate in vitro, Wnt signaling was downregulated less efficiently under osteogenic conditions when compared to either G-292 or TE-85 cells (Figures 7B and 7C). Expression of *AXIN2*, which has been previously shown to be downregulated during hMSC osteogenic and adipogenic differentiation (Liu et al., 2009), decreased during osteogenic differentiation in G-292 and TE-85 cells but was unaffected in

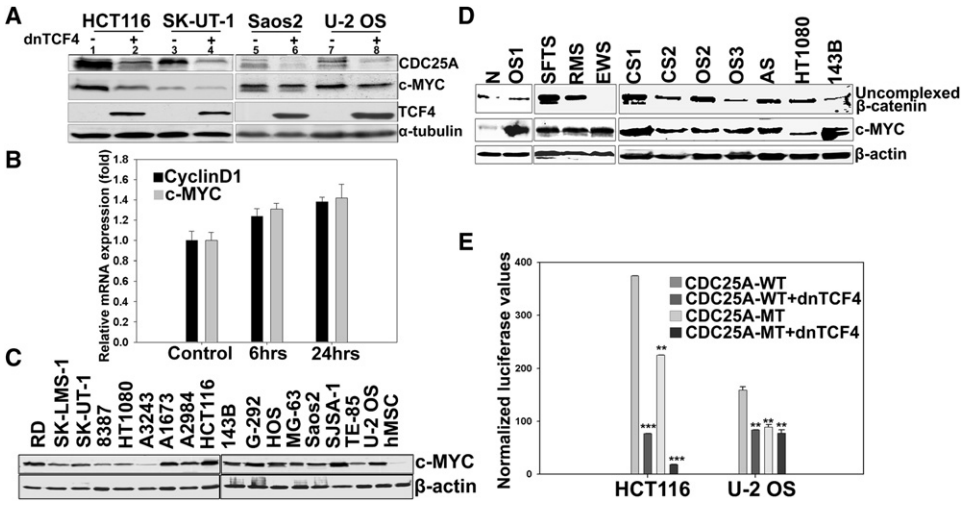

Figure 6. Lack of Correlation between Wnt Signaling and c-MYC Levels in Human Sarcomas

(A) Human sarcoma lines and HCT116 cells were stably transduced with dnTCF4, and proteins were analyzed by western blotting.

(B) Real-time PCR analysis of *c-MYC* and *CyclinD1* in hMSCs treated with Wnt3A as described in Figure 3A. The values were normalized to TBP and represented relative to those in untreated cultures (Control), which were set at one. Error bars indicate SD of mean values from triplicates and are representative of two independent experiments.

(C) Western blot analysis of human sarcoma cell lines for c-MYC protein.

(D) c-MYC protein expression in protein extracts from frozen sections of primary sarcoma tissues. N, normal tissue; CS, chondrosarcoma; AS, angiosarcoma. All other abbreviations for sarcomas are described in Figure 1. HT1080 and 143B lines were used as positive and negative controls for Wnt-activated sarcomas.

(E) HCT116 or U-2 OS cells were cotransfected with vector control, CDC25A-WT, or CDC25A-MT (see Figure 3F) luciferase reporter constructs (200 ng) and a dnTCF4 expression construct (400 μg). Luciferase values were measured 48 hr posttransfection and normalized to Renilla. Error bars indicate SD of mean values from triplicates and are representative of two independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ by Student's t test.

See also Figure S5.

U-2 OS cells (Figure 7D). Together, these results suggest that under differentiation-inducing conditions, high Wnt activity in osteosarcoma cells with osteogenic differentiation ability is downregulated by an intrinsic mechanism.

None of the Wnt-activated sarcoma lines analyzed showed evidence of multilineage differentiation capacity when exposed to osteogenic (Figure 7E) or adipogenic (Figure S6A) differentiation conditions in the absence or presence of Wnt downregulation. These results suggested that Wnt-independent aberrations might be responsible for the differentiation block. Frequent genetic aberrations observed in sarcomas include highly specific chromosomal translocations, as in the case of Ewing's sarcoma, synovial sarcoma, and alveolar rhabdomyosarcoma, and loss of function mutations of Rb and p53 observed in osteosarcomas (reviewed in Helman and Meltzer, 2003; Skubitz and D'Adamo, 2007). Thus, we sought to model genetic aberrations observed in osteosarcomas by ectopic expression of cyclin-dependent kinase 4 (CDK4) or dominant-negative p53 (dnp53), and oncogenic ras^{G12D} either alone or in combination in hMSCs. The expression of any one of the three genes in addition to hTERT, which did not itself block hMSC osteogenic differentiation, was sufficient to partially inhibit AP expression without exhibiting an obvious effect on mineralization as detected by AR staining (Figure S6B). However, the severity of osteogenic differentiation inhibition based on both reduced AP expression and mineralization increased with the number of oncogenes added. These findings argue that genetic alterations independent of Wnt activation may mask the ability to correlate Wnt activity with stemness in sarcomas.

DISCUSSION

MSCs can differentiate along osteogenic, adipogenic, chondrogenic, myogenic, and other connective tissue lineages in response to specific differentiation media (Pittenger et al., 1999). hMSCs have been shown to exhibit a low level of endogenous Wnt signaling, which decreases in response to differentiation stimuli (Li et al., 2005). Moreover, evidence that exogenous Wnt stimulation specifically increased hMSC proliferation and inhibited differentiation (Liu et al., 2009), properties commonly associated with tumor cells (Hanahan and Weinberg, 2000; Liu et al., 2009; van de Wetering et al., 2002), led us to explore whether Wnt upregulation might contribute to sarcomagenesis. Our present studies establish constitutive Wnt activation relative to levels observed in hMSCs in a diverse array of human sarcomas. In contrast to colon carcinomas, in which Wnt activation involves genetic aberrations in intracellular components, we established that Wnt activation in sarcomas commonly involves an autocrine mechanism as shown by the ability of antagonists, which act at the cell surface to block Wnt ligand/receptor interactions, to downregulate this signaling. *β-catenin* mutations were absent in any of the Wnt-activated sarcoma cell lines screened. However, we identified *APC* truncation mutations in two sarcoma lines that lacked an autocrine-activation mechanism. Of note, A2984 and SK-UT-1, like representative Wnt autocrine sarcoma lines, were growth inhibited in response to dnTCF4, which had no effects on Wnt negative sarcoma cells. These results establish the Wnt specificity of this inhibition and the essential role of Wnt signaling in driving the proliferation of sarcoma cells

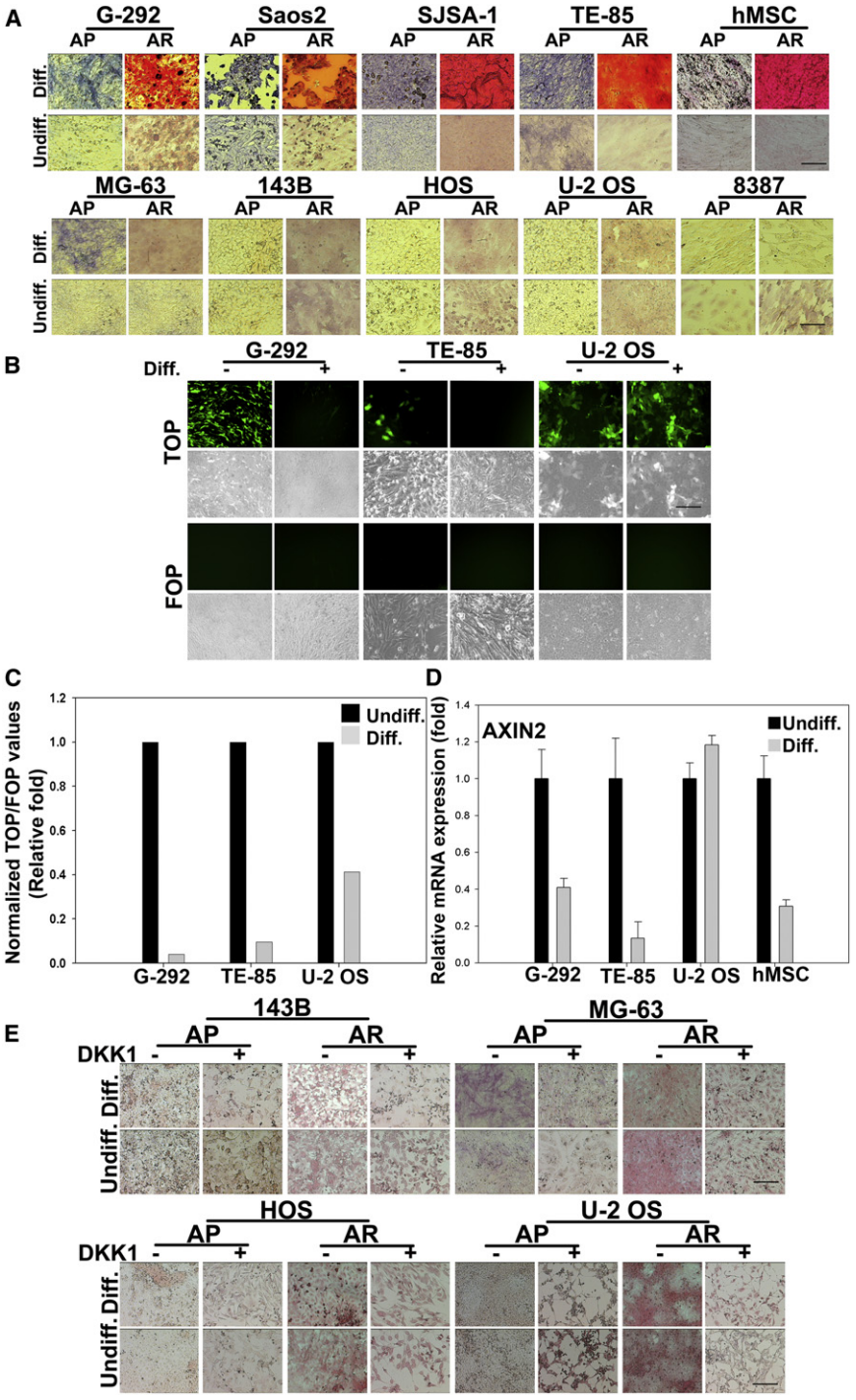

Figure 7. In Vitro Differentiation Properties of Wnt-Activated Human Sarcoma Cells

(A) Sarcoma lines were plated at 10,000 cells/well in 12-well plates and exposed to osteogenic differentiation media (Diff.) for 2 weeks, followed by fixation and staining for either AP (blue) or AR (red). Control cells were maintained in basal medium (Undiff.).

(B) Sarcoma lines stably expressing TOP- or FOP-GFP reporters were cultured as described in (A), and images were captured with a fluorescence microscope or by phase contrast.

(C) TOP-luciferase reporter activity in sarcoma cells cultured in basal or osteogenic differentiation media. Values are normalized to Renilla. Normalized values obtained for cells cultured in basal media (Undiff.) were set at one.

(D) Real-time PCR measurement of *AXIN2* using total RNA extracted from sarcoma cells or hMSCs cultured in the presence or absence of osteogenic medium for 2 weeks as described in Figure 2C. Normalized values obtained for cells cultured in basal media (Undiff.) were set at one. Error bars indicate SD of mean values from triplicates and are representative of two independent experiments.

(E) Sarcoma lines resistant to osteogenic differentiation were cultured in the presence or absence of osteogenic medium along with DKK1 (100 ng/ml) added every other day for 2 weeks and stained for AP and AR. Scale bars indicate 100 μm.

See also Figure S6.

activation was also identified in primary human sarcomas, which exhibited elevated uncomplexed β-catenin levels in more than 50% of samples analyzed from a wide array of sarcoma subtypes. There are reports implicating the hMSC as the cell of origin for Ewing's (Miyagawa et al., 2008; Tirode et al., 2007) and MFH/pleomorphic sarcomas (Matushansky et al., 2007), and we detected Wnt activation in 50% of Ewing's sarcoma cell lines. Of note, hMSC adipogenic differentiation is particularly sensitive to Wnt inhibition (Ross et al., 2000), and the lowest frequency of Wnt activation was observed in sarcomas classified histopathologically as liposarcomas. All of these findings are consistent with a model in which sarcomas commonly initiate from upregulation of autocrine Wnt signaling, and less frequently from mutations in intracellular components, in a MSC associated with its clonal expansion and accumulation of additional alterations that contribute to malignant transformation. However, our findings do not exclude the possibility that Wnt pathway activation occurs at some later stage of neoplastic progression and/or involves a committed progenitor rather than a stem cell.

in which the pathway is upregulated. Furthermore, Wnt autocrine activation contributes as significantly to the Wnt proliferative drive as an *APC* mutation, previously established as the initiating step in colon tumorigenesis (Bienz and Clevers, 2000; Korinek et al., 1997; Morin et al., 1997; Polakis, 1997; Roose et al., 1999).

The frequency of Wnt activation was greater than 90% in osteosarcomas and was at least 50% in synovial, Ewing's, rhabdomyo-, fibro-, and leiomyosarcoma cell lines. Constitutive Wnt

We observed that the sarcoma lines analyzed lacked multilineage differentiation capacity, even under the conditions in which

Wnt-signaling downregulation resulted in profound inhibition of proliferation. Thus, if the target for Wnt pathway activation were a mesenchymal stem/progenitor cell, Wnt-independent alterations must override the differentiation potential of such cells. In fact we found that oncogenic alterations of Rb, p53, or Ras pathways, observed in osteosarcomas (Kansara and Thomas, 2007), alone or in combination resulted in progressive inhibition of the ability of hMSCs to undergo osteogenic differentiation. These findings argue strongly that oncogenic alterations independent of Wnt pathway activation can impair the ability of a MSC to undergo lineage differentiation.

Mechanisms Involved in Activation of an Autocrine Wnt Loop in Human Sarcomas

There are reports in the literature of nuclear accumulation of β-catenin in a significant fraction (28%–57%) of synovial and uterine soft tissue sarcomas without exploration of possible mechanisms (Jung et al., 2008; Kildal et al., 2009; Ng et al., 2005). Our present findings of Wnt autocrine activation establish the molecular basis for nuclear accumulation of β-catenin in such tumors. In a mouse model generated by loss of function of WIF1, a Wnt antagonist, there was an increased incidence of radiation-induced sarcomas (Kansara et al., 2009), and these same authors identified epigenetic silencing of *WIF1* in some human osteosarcomas associated with upregulation of Wnt signaling (Kansara et al., 2009). In contrast, Cai et al. (2010) recently reported the inability to detect canonical Wnt activity in osteosarcomas analyzed. Our present findings demonstrate activation of this pathway by several different approaches in osteosarcoma lines studied by Cai et al. (2010). Thus, the failure of Cai et al. (2010) to detect Wnt pathway activation in human osteosarcomas may involve differences in assay sensitivity.

Our findings that sarcomas of diverse types exhibit autocrine canonical Wnt activation adds this tumor to those including breast, ovarian, and lung carcinoma exhibiting autocrine Wnt activation at high frequencies (Akiri et al., 2009; Bafico et al., 2004). We observed that relative to hMSCs, sarcomas often exhibited upregulated expression at the RNA level of canonical Wnt ligands, most commonly Wnt7b and Wnt10b. Sarcoma lines also frequently overexpressed LRP5, LRP6, or both receptors, with evidence of *LRP5* gene amplification in some. Epigenetic silencing of Wnt antagonists, including *FRP1*, *FRP2*, *FRP4*, *FRP5*, *DKK1*, and *DKK2*, was identified in some sarcoma lines as well. Thus, there appear to be several mechanisms by which autocrine Wnt signaling can be upregulated in human sarcomas, as demonstrated by the specific growth inhibition associated with LRP5 and LRP6 downregulation in HOS and A204 sarcoma lines, respectively. Our findings of more than one alteration in Wnt cell surface components in some autocrine tumor lines could imply continued selection for Wnt pathway activation by this mechanism.

Cell Context Differences in the Role of Wnt Target Genes in Tumor Cell Proliferation

Wnt signaling exerts its oncogenic functions via upregulation of genes involved in cell cycle, exemplified by the positive TCF/β-catenin transcriptional effects on *c-MYC* expression in carcinomas (Akiri et al., 2009; Bafico et al., 2004; van de Wetering et al., 2002). *c-MYC* is required for efficient activation of all cyclin/CDK complexes and, thus, is a vital player acting at multiple phases of the cell cycle (Mateyak et al., 1999). We observed that unlike HCT116 colon carcinoma cells in which c-MYC is responsible for Wnt-induced proliferation (He et al., 1998; van de Wetering et al., 2002), c-MYC was not transcriptionally regulated in a Wnt-dependent manner in Wnt-activated sarcomas. Instead, we identified *CDC25A* as a Wnt transcriptional target, as demonstrated by ChIP assay and β-catenin dependent activation of CDC25A reporter in sarcoma cells. Furthermore, we showed that CDC25A was an important mediator of Wnt-induced sarcoma cell proliferation both in vitro and in vivo. Although Wnt pathway downregulation in each sarcoma line analyzed led to reduced CDC25A transcript and protein levels, the level of CDC25A protein expression did not correlate perfectly with Wnt activation among the sarcoma lines analyzed, likely due to differences among tumor lines in CDC25A post-translational regulation (Boutros et al., 2007). Recently, it was reported that GSK3β, an effector kinase inhibited by Wnt ligand-induced stimulation, regulates CDC25A protein levels by proteasome-mediated degradation (Kang et al., 2008). Thus, GSK3β inhibition by autocrine Wnt activation could in theory lead to increased CDC25A protein stabilization. However, our results provide no evidence for Wnt-dependent regulation of CDC25A expression at the protein level by such a mechanism.

CDC25A is overexpressed in multiple cancers, including breast, ovarian, lung, colorectal, pancreatic, and head and neck cancer (reviewed in Boutros et al., 2007). It is inferred from animal studies that among the CDC25 family, CDC25A plays a nonredundant role in regulating cell cycle (Chen et al., 2001; Ferguson et al., 2005; Ray et al., 2007b). MMTV-targeted expression of CDC25A cooperated with neu or ras-induced mammary tumorigenesis, whereas mice hemizygous for *Cdc25A* exhibited prolonged latency of neu or ras-induced mammary tumors (Ray et al., 2007a; Ray et al., 2007b). *CDC25A* is also transcriptionally regulated by c-MYC as well as by E2F1 in mammalian cells (Galaktionov et al., 1996; Vigo et al., 1999). Given that *CDC25A* is a transcriptional target of both TCF/β-catenin, as shown here, and c-MYC (Galaktionov et al., 1996), the inhibition of proliferation induced by dnTCF4 in colon and other carcinomas may be mediated through CDC25A as well as c-MYC.

In summary our present studies show that the Wnt canonical upregulation by autocrine or, less frequently, by other mechanisms is common in human sarcomas of many subtypes. Furthermore, Wnt upregulation by autocrine activation or other mechanisms plays an important causal role in the proliferative drive of Wnt-activated sarcomas through a Wnt target gene, *CDC25A*. Over the past two and a half decades since the discovery of the involvement of activated tyrosine kinase receptor pathways in tumors, agents that specifically target these pathways have provided clinically useful adjuvants to standard chemo-irradiation therapies (reviewed in Zhang et al., 2009). The high prevalence of Wnt pathway activation in human sarcomas described here and the ability to identify Wnt pathway activation in primary sarcoma tissues make it reasonable to test whether naturally occurring Wnt antagonists such as DKK1 or FRP or recently reported small molecule Wnt inhibitors (Doghman et al., 2008; Huang et al., 2009) may complement standard agents in the treatment of this most common childhood malignancy.

EXPERIMENTAL PROCEDURES

Uncomplexed β-Catenin Analyses

Immunoprecipitation to detect uncomplexed β-catenin was performed as described previously (Bafico et al., 2004). Briefly, 1 mg of protein from whole-cell lysate was incubated with GST-E-cadherin/Glutathione-Sepharose beads (Amersham) for 1 hr at 4°C. Beads were pelleted and washed thrice in lysis buffer and resuspended in loading buffer for western blot analysis as described in Supplemental Experimental Procedures.

Human Sarcoma Tissues

Frozen sarcoma tissues were purchased from NCI's Cooperative Human Tissue Network or obtained through the Tisch Cancer Institute's Biorepository at Mount Sinai Hospital. Sarcoma tissues acquired from the Mount Sinai Hospital used in this study were de-identified prior to analysis. The use of human tissues was approved by the Institutional Review Board. Frozen tissues were embedded in Optimal Cutting Temperature (OCT), and 20 sections of 20 μm each were used. Tumor tissues were analyzed histologically to confirm the presence of at least 70% tumor cells. Excess OCT was removed, and tissues were rinsed thrice in cold PBS. Buffer containing 1% NP-40, 150 mM sodium chloride, and protease inhibitors was used for protein solubilization.

Microarray

Total RNA extracted from hMSCs was used for gene expression analysis on the GeneChip Human Genome U133A 2.0 Array (Affymetrix). Hybridization and data acquisition were conducted at Mount Sinai's Microarray Shared Research Facility according to the manufacturer's protocol. Data were analyzed using GeneChip Operating Software (Affymetrix) and Ingenuity's Pathway Analysis software.

In Vivo Tumorigenicity Assay

HT1080 cells stably expressing firefly luciferase were transduced with vector control, dnTCF4, or CDC25A shRNA and selected as described for colony-forming assay. Firefly luciferase was cloned in a lentiviral vector carrying a neomycin marker gene. Standard procedures were used to clone the full-length CDC25A cDNA into a PGK promoter-driven lentiviral construct containing a blasticidin marker gene. Cells expressing both dnTCF4 and CDC25A were selected in medium containing 1 μg/ml puromycin and 5 μg/ml blasticidin (Invitrogen). Around 1×10^6 cells were resuspended in PBS and mixed with 50% Matrigel (BD Biosciences) prior to subcutaneous inoculation at two sites in 6-week-old athymic nude mice (NCI). All animal experiments were approved and performed according to the relevant regulatory standards set by Mount Sinai's Animal Care and Use Committee. Mice were anesthetized using isoflurane, and in vivo imaging was performed using the Xenogen IVIS-200 Imaging System at Mount Sinai's shared in vivo imaging facility.

Osteogenic and Adipogenic Differentiation Assay

Cells were transferred to 24-well plates and cultured in either basal medium (DMEM, 10% FBS, 100 U/m P/S) or osteogenic medium consisting of DMEM, 10% FBS, 100 U/m P/S supplemented with 50 μg/ml ascorbic acid (Biochemika), 10 mM β-glycerol-2-phosphate (Sigma), and 1 μM dexamethasone (Sigma). Cells were subjected to differentiation conditions for around 2 weeks and processed for staining as described previously (Liu et al., 2009). Adipogenic differentiation was performed as previously described (Liu et al., 2009).

Statistical Analyses

Statistical significance was determined with Student's t test or one-way analysis of variance (ANOVA) in the case of more than two comparisons (calculated with GraphPad Prism 5 software).

ACCESSION NUMBERS

Microarray data generated in this study have been deposited at the NCBI Gene Expression Omnibus (http://ncbi.nlm.nih.gov/geo/) with the accession number GSE27313.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at doi:10.1016/j.ccr.2011.03.010.

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