Targeting the Wnt Pathway in Synovial Sarcoma Models

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ABSTRACT

Synovial sarcoma is an aggressive soft-tissue malignancy of children and young adults, with no effective systemic therapies. Its specific oncogene, SYT–SSX (SS18–SSX), drives sarcoma initiation and development. The exact mechanism of SYT–SSX oncogenic function remains unknown. In an SYT–SSX2 transgenic model, we show that a constitutive Wnt/β-catenin signal is aberrantly activated by SYT–SSX2, and inhibition of Wnt signaling through the genetic loss of β-catenin blocks synovial sarcoma tumor formation. In a combination of cell-based and synovial sarcoma tumor xenograft models, we show that inhibition of the Wnt cascade through coreceptor blockade and the use of small-molecule CK1α activators arrests synovial sarcoma tumor growth. We find that upregulation of the Wnt/β-catenin cascade by SYT–SSX2 correlates with its nuclear reprogramming function. These studies reveal the central role of Wnt/β-catenin signaling in SYT–SSX2-induced sarcoma genesis, and open new venues for the development of effective synovial sarcoma curative agents.

SIGNIFICANCE: Synovial sarcoma is an aggressive soft-tissue cancer that affects children and young adults, and for which there is no effective treatment. The current studies provide critical insight into our understanding of the pathogenesis of SYT–SSX-dependent synovial sarcoma and pave the way for the development of effective therapeutic agents for the treatment of the disease in humans. Cancer Discov; 3(11); 1286–1301. © 2013 AACR.

INTRODUCTION

Synovial sarcoma is a high-grade soft-tissue cancer most commonly diagnosed in patients between 15 and 35 years of age. Synovial sarcoma survival rates remain low (10%–30% 10-year survival; refs. 1–3), thus necessitating effective, specific therapies. Synovial sarcoma is associated with a specific t(x;18)(p11.2;q11.2) chromosomal translocation that generates the SYT–SSX (also known as SS18–SSX) fusion, a potent oncogene believed to play a critical role in synovial sarcoma initiation and progression. How SYT–SSX induces tumorigenesis is still unknown.

Synovial sarcoma belongs to a group of mesenchymal tumors that arise in multipotent stem/precursor cells, hence their varied nature and locations (4, 5). We recently reported that the synovial sarcoma oncogene SYT–SSX2 reprograms the differentiation of mesenchymal stem/precursor cells (6). This aberrant reprogramming results from SYT–SSX2 tight associations with, and antagonistic effect on, components of Polycomb and switch/sucrose nonfermentable (SWI/SNF) (7, 8), the two chromatin-modifying complexes that temporally coordinate multipotency and differentiation in development (9). SYT–SSX2 directly targets Polycomb-silenced lineage-specific genes (10) in mesenchymal precursor cells. In the same studies, we observed that in addition to reprogramming differentiation, SYT–SSX2 activated several signaling pathways that ordinarily control stem cell maintenance and fate allocation, including the Wnt/β-catenin pathway (6).

We first discovered a role of SYT–SSX2 in β-catenin regulation when the Wnt mediator was seen accumulated in the nucleus of SYT–SSX2-expressing cells, with a substantial increase in β-catenin reporter activity (11). β-catenin and SYT–SSX2 coexisted in active nuclear complexes, and a functional link between the two proteins was established when SYT–SSX2 depletion in primary human synovial sarcoma cells led to a marked attenuation of β-catenin nuclear accumulation (11).

Wnt/β-catenin signaling controls embryogenesis, and its untimely activation in adult organisms frequently leads to tumorigenesis (12, 13). Whether activated by genetic lesions or epigenetic mechanisms, an aberrant Wnt/β-catenin signal is often considered a marker for poor disease prognosis, and is implicated in tumor progression and invasiveness. Deregulation of Wnt/β-catenin signaling in synovial sarcoma was described in several studies (>300 tumors examined) where β-catenin accumulation was detected in the majority of tumors and was predictive of poor survival (14–16). A study showing β-catenin nuclear localization in primary (41%) and metastatic (70%) synovial sarcoma tumors (82 total) indicated that its misregulation occurs early in tumorigenesis and is maintained as the cancer progresses (17). Activating genetic mutations of Wnt pathway components in synovial sarcoma tumors are rare (18–20), suggesting that constitutive activation of Wnt/β-catenin in synovial sarcoma is caused by an upstream signal. Analysis of a diverse group of human
sarcoma tumors and cell lines representing the major subtypes revealed an active Wnt signal, suggesting a link between Wnt signaling and sarcoma growth (21). Despite the association between Wnt signaling and synovial sarcoma, there has not been a clear demonstration of a direct link between SYT–SSX expression, Wnt/β-catenin activation, and the role of Wnt signaling in initiation and/or progression of synovial sarcoma in vivo.

In the current studies, we investigated the role of Wnt/β-catenin signaling in synovial sarcoma development, using synovial sarcoma cultures, synovial sarcoma tumor xenografts, and a SYT–SSX2 transgenic model where tumors develop with histologic and gene expression features that recapitulate those of human synovial sarcoma tumors (22). These analyses led to three novel and significant findings. First, we show that SYT–SSX2 activates Wnt/β-catenin signaling through the domains that execute its cellular reprogramming function. Second, we provide evidence that the constitutive Wnt/β-catenin signal activated by the SYT–SSX2 oncogene is necessary for synovial sarcoma tumor growth. Finally, we show that the Wnt inhibitor, pyrvinium, which activates CK1α, and a pyrvinium functional equivalent represent promising therapeutic agents for the treatment of synovial sarcoma. Altogether, our results illustrate the critical role of Wnt/β-catenin signaling in synovial sarcoma pathogenesis and growth, present the Wnt/β-catenin pathway as an appropriate target for synovial sarcoma therapy, and illuminate a novel venue for the development of effective agents for difficult-to-treat (unresectable) sarcomas.

**RESULTS**

**β-Catenin Is Deregulated in Synovial Sarcoma, and Its Function Is Required for Tumor Development in SYT–SSX2 Transgenic Mice**

To determine the importance of Wnt/β-catenin signaling in synovial sarcoma development, we examined tumor growth in a β-catenin-deficient background. These studies were conducted in the conditional SYT–SSX2 transgenic murine model (22) where SYT–SSX2 is expressed in the Myf5-positive myoblasts (SSM2+/Myf5-Cre+), and tumors that mimic human synovial sarcoma develop with 100% penetrance, most frequently in the ribs at the bone/cartilage junctions. To create a β-catenin-null background, SYT–SSX2 transgenic mice (SSM2+) were mated in two steps (Supplementary Fig. S1A) with mice carrying a floxed β-catenin locus (23) to produce double-mutant SSM2+ mice with one or two targeted β-catenin allele(s) (SSM2+/B-CATδ/− and SSM2+/B-CATδ/−/B-CATδ/−). The resultant progeny were bred with Myf5-Cre+ mice or with Myf5-Cre+ mice bearing a floxed allele of β-catenin (B-CATδ/−/Myf5-Cre+). The final outcome (Supplementary Fig. S1A) is the production of two study groups of triple-mutant mice: SG2 (SSM2+/B-CATδ/−/Myf5-Cre+) and SG3 (SSM2+/B-CATδ/−/B-CATδ/−/Myf5-Cre+), where SYT–SSX2 expression and β-catenin knockout occurred in the Myf5 myoblasts. As reported previously (22), by 3 months of age, all the SG1 (SSM2+/Myf5-Cre+) mice had developed on average three to four tumors each, with 100% penetrance. The synovial sarcoma tumors most frequently arose in the intercostal muscles or near the vertebral, and occasionally in the skeletal musculature of the neck and limbs. The tumors were small in size, well defined, vascularized, and easily detected by eye (Supplementary Fig. S1B) or by GFP imaging. On the basis of these criteria, tumor analyses were conducted in 3-month-old mice in the three experimental groups. Comparison of tumor formation in 8 of SG1, 14 of SG2, and 13 of SG3 mice revealed striking differences in the number of detectable tumors among the three groups, indicating a dependence of sarcoma growth on β-catenin (Fig. 1A). Although β-catenin heterozygous (SG2) mice still developed tumors, the overall tumor load was significantly less than that observed in the SG1 mice (Fig. 1B). The most significant finding, however, was the complete absence of visible tumors in 10 of the SG3 mice (SSM2+/B-CATδ/−/Myf5-Cre+). This is a dramatic result, given the 100% frequency of tumor formation observed in SG1 mice. The appearance of tumors in the remaining three SG3 mutants prompted us to conduct an immunohistochemical analysis of the sarcoma tissues to determine the status of β-catenin. As expected, histologic staining of all resected tumors showed sheets of malignant cells (blue nuclei) surrounding the muscle fibers (pink structures; Fig. 1C, left column, black arrows), consistent with their origin from Myf5 myoblasts. Most importantly, all the sarcomas of the SG1 and SG2 mice displayed a strong nuclear and/or cytoplasmic β-catenin signal, suggesting deregulation of Wnt/β-catenin signaling in the tumors (Fig. 1C, right images, compare with IgG control; Fig. 2A). β-catenin nuclear localization in the synovial sarcoma tumors was further confirmed by immunofluorescent staining (Fig. 1D). β-catenin accumulation was heterogeneous and easily detected in 60% to 98% of tumor nuclei. A key finding was the strong β-catenin nuclear signal seen in the few SG3 tumors (Fig. 1C, bottom right image). Myf5 expression in these SG3 tumors confirmed their origin from myoblasts (Supplementary Fig. S2). In SG1 and SG3 tumors, β-catenin accumulated to equivalent levels, strongly suggesting β-catenin escape from the Cre recombinase in the SG3 mice. This is consistent with incomplete β-catenin depletion by Cre in other systems (24, 25). Our results from the SG2 and SG3 mice strongly suggest that complete loss of β-catenin is required for the total inhibition of SYT–SSX2-induced sarcomas (Fig. 2A). Thus, in a conditional SYT–SSX2 transgenic model where expression of the fusion is sufficient for malignant transformation of muscle precursors, depletion mutations of the β-catenin locus negatively affect development of detectable synovial sarcoma tumors.

Production of the double- and triple-mutant mice followed normal rules of Mendelian inheritance. One hundred fifty mice with intermediate single and double mutations served as control littersmates for the SG2 and SG3 mice (Supplementary Fig. S1A). Mice bearing the SYT–SSX2 (SSM2+) or Myf5-Cre (Myf5-Cre+) transgenes or the targeted β-catenin loci (B-CATδ/−, B-CATδ/−/−), as well as double mutants with the SYT–SSX2 transgene and targeted β-catenin loci (SSM2+/B-CATδ/−/−/Myf5-Cre+) and SG3 (SSM2+/B-CATδ/−/−/B-CATδ/−/−/Myf5-Cre+), developed normally and were tumor free. Normal development of the B-CATδ/−/−/Myf5-Cre+ and B-CATδ/−/−/Myf5-Cre+ controls was critical, as these mice were used in the final production of SG2 and SG3 mice. Both double mutants were fertile, producing normal-size litters. Histologic analysis of their rib cage revealed normal musculature (Fig. 2B), thus showing that targeting one
Figure 1. β-catenin knockout inhibits synovial sarcoma (SS) tumor growth in SYT-SSX2 transgenic mice. A, histogram represents total number of tumors in SG1 (SSM2+/Myf5-Cre+), SG2 (SSM2+/B-CATfl+/−/Myf5-Cre+), and SG3 (SSM2+/B-CATfl+/+/Myf5-Cre+) mice. B, dot plot tabulating number of tumors in the SG1, SG2, and SG3 mice, with median value bar. P values show significance of the difference between each pair of the experimental groups. C, left column, hematoxylin and eosin (H&E) staining of tumors (black arrows: blue nuclei) in the intercostal muscle (pink fibers) of SG1 (top row, SSM2+) and one of the three SG3 (bottom row, SSM2+/B-CATfl+/+) mice that developed tumors. Middle column, control mouse immunoglobulin G (IgG) immunostaining. Right column, β-catenin immunostaining. Control IgG and β-catenin stainings were conducted on serial tumor sections present on the same slide. Scale bars of all images are 99 μm. D, β-catenin immunofluorescent staining (red) in tumors from SG1 (SSM2+) and SG3 (SSM2+/B-CATfl+/+) mice. Mouse IgG served as negative control. DAPI (blue) stains nuclei. Merged blue and red images show β-catenin nuclear localization.
Barham et al.

Figure 2. Integrity of the rib musculature in SYT–SSX2 transgenic mice. A, H&E staining (left and middle images) of an intercostal SG2 tumor (black arrow, pink muscle fibers) near the rib cartilage (black arrowheads). Right, β-catenin immunostaining in the SG2 tumor. Scale bars on middle and right images are 99 μm. B, H&E staining of the rib musculature. Top row, left and middle images: SG2 tumor (black arrow, blue nuclei), infiltrating muscle fibers (pink) near the rib bone/cartilage. Top right image: normal rib musculature in a tumor-free SG3 mouse. Bottom row: normal rib musculature in a β-catenin–null knockout mouse (left image), a heterozygous β-catenin knockout mouse (middle image), and a SYT–SSX2/B-CAT double mutant (right image). Black arrowheads denote cartilage/bone junction. Except the top left image, all displayed scale bars are 99 μm.

or both β-catenin alleles in the Myf5 lineage did not adversely affect muscle development. Immunohistochemical analysis in β-catenin–deficient mice confirmed normal Myf5 myoblast development (Supplementary Fig. S3; ref. 26), indicating that upon β-catenin loss, the myoblasts develop resistance to SYT–SSX2 oncogenicity.

Taken together, these results show that deregulation of β-catenin is a persistent feature of SYT–SSX2-induced synovial sarcoma tumors and is necessary for their development.

Inhibition of the Wnt Cascade Attenuates SYT–SSX2-Induced β-Catenin Nuclear Accumulation and Synovial Sarcoma Tumor Growth

To begin to understand the mechanism of SYT–SSX2-induced β-catenin deregulation in the SG1 (SSM2+/Myf5-Cre+) tumors, we asked whether it originated from upstream activation of the Wnt cascade. For in vitro analysis, we chose the C2C12 myoblasts, as they are equivalent to the Myf5 myoblasts (27) where the SG1 tumors developed. C2C12 transduction with retroviral SYT–SSX2 (∼90% infection rate) resulted in nuclear β-catenin accumulation in the majority of SYT–SSX2 infectants (Fig. 3A, SYT–SSX2) and a concomitant increase in β-catenin reporter activity (Fig. 3B). To locate the origin of the β-catenin–activating signal, we sequentially infected C2C12 myoblasts with SYT–SSX2- and DKK1- (potent inhibitor of the Wnt receptor; ref. 28) expressing retroviruses, at 80% to 90% rates. DKK1 expression significantly reduced SYT–SSX2-induced β-catenin nuclear accumulation (Fig. 3A, SYT–SSX2 + DKK1) and transcriptional activity (Fig. 3B). In contrast, the expression of neither empty vector (POZ and/or
**Targeting Wnt in Synovial Sarcoma**

**Figure 3.** DKK1 attenuates β-catenin nuclear localization and signaling. **A,** immunostaining of C2C12 myoblasts transduced with empty retroviral vectors: POZ and LZRS, and those expressing SYT-SSX2 and DKK1. DKK1 (green) and β-catenin (red) were detected with a specific polyclonal and monoclonal antibody, respectively. Numbers represent average percent of SYT-SSX2/DKK1-expressing cells, exhibiting nuclear β-catenin, ± SDs (n = 3). Five hundred nuclei were counted for each vector. **B,** TOP-FLASH activity in SYT-SSX2-expressing C2C12 cells transduced with incremental amounts of DKK1 cDNA. Histogram represents fold activation over vector. Error bars are SDs (n = 3). P values were calculated relative to SYT-SSX2.

LZRS altered β-catenin localization or activity. These results strongly indicate that in C2C12 cells, a Wnt signal activated by SYT-SSX2 at the receptor level is responsible for β-catenin nuclear accumulation.

To verify whether similar receptor-mediated Wnt signaling is activated in the SYT-SSX2-induced tumors and affects their growth, we expressed DKK1 in murine synovial sarcoma model tumor cells (mSS) isolated from SG1 tumors. In the mSS cells, ectopic DKK1 inhibited intrinsic β-catenin/T-cell factor (TCF) activity in a dose-dependent fashion (Fig. 4A), thereby revealing a constitutive Wnt signal. Subcutaneous injection of naïve or vector-expressing mSS cells in nude mice (5 mice/group) led to the formation of measurable tumors in all the mice (100%) within 4 days of growth. Final tumor sizes were recorded 2 weeks after implantation (Fig. 4B). DKK1 expression led to significant reduction in mSS tumor growth (Fig. 4B). These data suggest that synovial sarcoma tumors depend on an intrinsic Wnt signal for their growth. Tissue histology revealed vascularized tumors with monophasic morphology typical of SYT-SSX2-expressing synovial sarcomas (Fig. 4C, left images). One noticeable feature was the appearance of hypocellular centers in the DKK1-expressing tumors (Fig. 4C, right images, arrows). Fluorescent imaging showed no apoptotic features. However, marked attenuation of Ki-67 expression in the DKK1-SS tumors (Fig. 4D) indicated proliferation arrest and suggested that Wnt signaling controlled synovial sarcoma tumor growth through its proliferative function. In conclusion, these results show that an aberrant, receptor-mediated Wnt/β-catenin signal, activated upon SYT-SSX2 expression, persists in synovial sarcoma tumors, and is necessary for their growth.
Figure 4. DKK1 inhibits synovial sarcoma tumor growth in nude mice. A, histogram represents ratios of TOP-FLASH (T) activity in mSS cells transfected with increasing amounts of DKK1 cDNA, over empty vector. Error bars indicate SDs (n = 3). P values were calculated relative to empty vector (T, 0). FOP-FLASH (F) was baseline activity. B, dot plot comparing tumor volumes in naïve, LZRS vector-, and LZRS-DKK1–infected mSS cells, with median value bars. A Dunn post hoc test showed significant difference between DKK1 and naïve or vector synovial sarcoma tumors. Naïve and vector were not significantly different. Inset, immunoblot showing stable DKK1 expression in mSS cells implanted in nude mice. Equivalent amounts (10 μg) of protein lysate were loaded in each lane. Doublet reflects DKK1 posttranslational modification. C) H&E staining of naïve, vector, or DKK1 tumors. Arrows indicate hypocellular centers. Scale bars on all images are 99 μm. D) Ki-67 (green, white arrowhead) immunofluorescent staining in vector and DKK1 tumors. DAPI staining; double arrowheads reveal paucity of nuclei in the hypocellular centers of DKK1 tumors. Right histogram compares Ki-67 expression (counted visually) in the vector and DKK1 tumors. Error bars indicate SDs (n = 3). P value was calculated relative to vector synovial sarcoma.
Targeting Wnt in Synovial Sarcoma

Wnt/β-Catenin Inhibition by Small Molecules Blocks Synovial Sarcoma Growth

The importance of an active Wnt/β-catenin signal in the development of SYT-SSX2-driven tumors led us to explore the clinical benefits of inhibiting Wnt signaling in synovial sarcoma. Pyrvinium pamoate is an anthelmintic that potently inhibits the Wnt/β-catenin pathway through axin stabilization and destabilization of β-catenin and pygopus (29). Pyrvinium activates the CK1ε kinase, and, through its nuclear effects, it bypasses surface signaling (29, 30).

We began by examining the effects of pyrvinium on SYT–SSX2-dependent β-catenin deregulation in C2C12 myoblasts. A 2-day treatment with incremental doses of pyrvinium induced gradual β-catenin nuclear exit and its accumulation at intercellular junctions (Supplementary Fig. S4A; white arrows). In addition, pyrvinium significantly reduced β-catenin reporter activity in SYT–SSX2-transduced cells (Fig. S4B). These results led us to test the effects of pyrvinium on mSS cells. At concentrations similar to those used with C2C12 cells, pyrvinium abrogated mSS cell growth (Supplementary Fig. S4C) and significantly reduced their constitutive β-catenin activity (Supplementary Fig. S4D).

For in vivo studies, we used SSTC-104, a functional equivalent of pyrvinium (StemSynergy Therapeutics, Inc). SSTC-104 functions through a mechanism similar to that of pyrvinium (activation of CK1ε; StemSynergy Therapeutics, Inc.), with the advantage of being well tolerated (LD₅₀ ~ 150 mg/kg) at high doses in live mice. SYO-1 and HS-SY-II are two human synovial sarcoma cell lines that express SYT–SSX2 and SYT–SSX1, respectively (31). Protein analysis in SYO-1 and HS-SY-II cells showed phosphorylation of the N-terminal serines and threonine that regulate β-catenin stability (Supplementary Fig. S5A). We also showed the expression of full-length adenomatous polyposis coli (APC) (Supplementary Fig. S5B) in both synovial sarcoma cell lines. The β-catenin (CTNNB1) N-terminal domain (Exon 3) and the APC mutation cluster region are 4 SSM2+Myf5-Cre+ mice and the other 4 non–tumor forming mice (Control) treated with SSTC-104. Comparative analysis of the three study groups at the end of the treatment period showed that SSTC-104–treated nude mice displayed clear perfusion deficits, and histologic examination of their skin, an organ dependent on Wnt signaling, showed normal structure (Supplementary Fig. S5A). This result suggests that Wnt signaling is more sensitive to inhibition in synovial sarcoma tumors than in the normal Wnt-regulated tissues (e.g., skin and gastrointestinal tract).

SSTC-104 was next tested for its effects on synovial sarcoma tumors in the SSM2+Myf5-Cre+ (SG1) model. At 3 months, the SYT–SSX2 transgenic mice had developed tumors with 100% efficiency. This age was marked as the treatment endpoint, and a group of 4 SSM2+Myf5-Cre+ mice were injected with SSTC-104 at 3 mg/kg. Treatment was begun 12 days before the mice reached 3 months, and SSTC-104 was administered every 2 days for a total of six injections. The study included two control groups, one comprised 4 vehicle-treated SSM2+Myf5-Cre+ mice and the other 4 non–tumor forming Myf5-Cre+ mice (Control) treated with SSTC-104. Comparative analysis of the three study groups at the end of the 12 days revealed a significant reduction in the number of visible/measurable tumors and overall tumor load (Fig. 6E, top and bottom graph, respectively) in the SSTC-104–treated SSM2+Myf5-Cre+ mice relative to vehicle controls. The SSTC-104–treated Myf5-Cre+ (Control) mice showed no obvious signs of drug-related side effects, as they maintained led to significant loss of nuclear β-catenin (Fig. 5E and Supplementary Fig. S7B), and marked morphologic changes with enhanced spreading, cytoplasmic protrusions, and β-catenin accumulation at intercellular junctions (Fig. 5F and Supplementary Fig. S7C; white arrowheads).

Thus, biochemical (DKK1 expression, LR6 deletion) inhibition and pharmacologic (pyrvinium, SSTC-104) inhibition of the Wnt cascade in synovial sarcoma cells exert parallel effects in inducing nuclear β-catenin exit and enhancing the β-catenin pool associated with stronger adhesion.

To verify whether SSTC-104 exerts similar effects on synovial sarcoma growth in vivo, HS-SY-II–derived tumors were treated with SSTC-104 over a period of 8 days. HS-SY-II cells were implanted subcutaneously in nude mice and two different doses of SSTC-104 (1.5 and 3 mg/kg) were injected intraperitoneally on days 4 and 8 postimplantation. Tumors were measured on both injection days with a final measurement on day 12. Six mice were used for each experimental group and for the group treated with vehicle. Comparison with control mice revealed significant reduction in tumor size after the first injection with either dose of SSTC-104 (Fig. 6A, left and middle graph). This growth effect was maintained by the second injection until the end of treatment (Fig. 6A, right graph).

Immunohistologic analysis of tumors resected from SSTC-104–treated mice displayed wide clear patches with β-catenin-free nuclei, in contrast with the homogeneous β-catenin staining in control tumors (Fig. 6B; double and single arrowheads). Fluorescence imaging showed extensive loss of nuclear β-catenin in the hypocellular centers (Fig. 6C; white arrows). Importantly, markedly decreased Ki-67 levels were measured on both injection days with a final measurement (Fig. 6D). These results are reminiscent of DKK1-expressing synovial sarcoma tumors and show an in vivo effect of SSTC-104 on synovial sarcoma growth. Importantly, SSTC-104-treated nude mice displayed no adverse side effects, and histologic examination of their skin, an organ dependent on Wnt signaling, showed normal structure (Supplementary Fig. S6A). This result suggests that Wnt signaling is more sensitive to inhibition in synovial sarcoma tumors than in the normal Wnt-regulated tissues (e.g., skin and gastrointestinal tract).
Figure 5. SSTC-104 and LRP6 depletion effects on β-catenin and synovial sarcoma cells. A, left histogram shows growth of SSTC-104–treated SYO-1 cells relative to vehicle (dimethyl sulfoxide; DMSO) after a 2- or 4-day treatment. Right histogram shows percentage of SSTC-104– and vehicle-treated cells containing nuclear β-catenin. Seven hundred nuclei were counted in each group. Error bars represent SDs (n = 2). P values were calculated relative to vehicle. B, immunoblot of dephosphorylated β-catenin in SSTC-104–treated SYO-1 cells. Numbers represent ratios of signal intensities over control (V = vehicle). Tubulin served as a loading control. C, β-catenin immunofluorescent staining (green) in SSTC-104–treated cells. White arrows indicate large cytoplasmic extensions with multiple intercellular junctions. Right image (high magnification) showcases β-catenin at intercellular junctions. DAPI stains nuclei. β-catenin and DAPI images were merged for colocalization. D, immunoblot shows LRP6 levels in SYO-1 cells transfected with LRP6 nontargeting (NT) and targeting (Si-LRP6-1 and Si-LRP6-2) siRNAs. Numbers show relative intensities (NT is 1). Tubulin served as loading control. E, histogram shows percent of siRNA-transfected SYO-1 cells containing nuclear β-catenin. Five hundred nuclei were counted in each group. Error bars represent SDs (n = 2). P values were calculated relative to NT. F, β-catenin immunofluorescent staining (green) in SYO-1 cells transfected with NT, Si-LRP6-1, and Si-LRP6-2 oligomers. Merged (merge) β-catenin and DAPI images show nuclear (or lack of) β-catenin. White arrowheads in Si-LRP6-1 and Si-LRP6-2 (magnified) images show neurite-like cytoplasmic protrusions connecting cells over long distances at multiple points of contact.
Figure 6. SSTC-104 in vivo effects in synovial sarcoma tumors. A, the three histograms represent tumor volumes on the first, fourth, and eighth day of SSTC-104 treatment. Vehicle is 15% DMSO in PBS solution. Error bars denote SDs. P values were calculated relative to vehicle. B, β-catenin immunostaining in vehicle-treated (top row; increasing magnification) and SSTC-104–treated (middle and bottom row) tumors. Double arrowheads: areas denuded of β-catenin. Single arrowheads: clear nuclei. C, immunofluorescent staining of β-catenin (red) in vehicle-treated tumors (top row), and in the hypocellular centers of SSTC-104–treated tumors (bottom row, white arrows). DAPI (not shown) and red images were merged (merge). Scale bar is 50 μm. D, histogram shows Ki-67 scores in SSTC-104– and vehicle-treated tumors. Five hundred nuclei were counted in each group. Error bars represent SDs. P values show significance of difference between each pair of groups. Bottom image panel, immunofluorescent staining of Ki-67 (green) in SSTC-104– and vehicle-treated tumors. DAPI stains nuclei. Scale bar is 50 μm. E, top histogram shows number of tumors detected in SSTC-104–treated Control and SSM2+/Myf5-Cre+ mice, and vehicle-treated SSM2+/Myf5-Cre+ mice. P value was calculated relative to vehicle. Error bars represent SDs. Lower histogram shows combined tumor volumes as tumor load in SSM2+/Myf5-Cre+ mice treated with vehicle or SSTC-104. Error bars represent SDs. P value was calculated relative to vehicle.
their healthy weight (Supplementary Fig. S8C) and normal structure of their intestinal crypts, which depends on Wnt/β-catenin signaling (Supplementary Fig. S8B).

Studies in the synovial sarcoma genetic model, combined with results from the synovial sarcoma tumor xenografts and pharmacologic models, suggest that the Wnt/β-catenin pathway is an important avenue for synovial sarcoma treatment in humans.

Insights into the Mechanism of Wnt/β-Catenin Pathway Activation by SYT–SSX2: Importance of the Domains That Program Differentiation

To elucidate the mechanism of Wnt/β-catenin pathway deregulation by SYT–SSX2, we conducted deletion analysis of SYT–SSX2 to identify the distinct regions involved in β-catenin nuclear localization and activation. This analysis revealed two functional modules, the carboxy (C)-terminal repressive domain (SSXR: amino acids 45–78 of SSX; 2), and the first 40 amino acids of the SYT amino (N)-terminal region (Fig. 7A and B and data not shown). We previously showed that the SSXR mediates SYT–SSX2 association with Polycomb components and disrupts their gene silencing function (7). The antagonistic effect on Polycomb silencing by SYT–SSX2 is the basis for its effects on reprogramming mesenchymal stem cell differentiation (6, 10). In the current analyses, we found that a deletion mutation in SSXR (SXdl9) that abrogates β-catenin nuclear localization and activation (Fig. 7 and Supplementary Fig. S9A) reduces levels of the Wnt/β-catenin targets cyclin D1 and Myc (Supplementary Fig. S9C) and reverses the myogenesis block induced by SYT–SSX2 in C2C12 cells (Supplementary Fig. S9B; ref. 6). These results reveal a structural link between β-catenin activation and differentiation reprogramming by SYT–SSX2. The 40 N-terminal residues of SYT–SSX2 contain the binding sites of the p300 acetyltransferase and the SWI/SNF ATPase BRG1, two components of coactivating and chromatin-modifying complexes that aid SYT–SSX2 in gene expression regulation (34, 35). Although removal of the entire N-terminal segment (residues 1–40) led to a significant decrease in β-catenin nuclear accumulation and signaling (Fig. 7A and B; SXdl40), removal of the first 20 amino acids or residues 15–44 of SYT–SSX2 abolished β-catenin reporter activity without impairing its nuclear accumulation (Fig. 7A and B; SXdl20 and SXdl15-44). Thus, our SYT–SSX2 deletion mutation data dissociate β-catenin nuclear localization from its transcriptional activation. Together, these results indicate that SYT–SSX2 activates the Wnt/β-catenin pathway through the domains that mediate its function as a coregulator of gene expression.

To assess the extent of Wnt pathway upregulation by SYT–SSX2, we analyzed two previously mined SYT–SSX2 gene expression arrays (6) conducted in C2C12 myoblasts and human bone marrow–derived mesenchymal stem cells (hMSC) that also displayed β-catenin nuclear localization upon SYT–SSX2 expression (Supplementary Fig. S9D). The analysis uncovered an autocrine Wnt/β-catenin loop upregulated by SYT–SSX2 (Supplementary Tables S1 and S2), comprising an extensive array of genes representing components and regulators of the Wnt cascade (40 genes, Supplementary Table S1), as well as known β-catenin/TCF downstream targets (147 genes; Supplementary Table S2). Comparative analysis of both arrays with transcriptomes of 40 human synovial sarcomas included in two independent studies (ref. 36: 8 tumors; ref. 37: 32 tumors) revealed a substantial overlap. The two human synovial sarcoma arrays contained a total of 113 Wnt component and target genes, 69 (61%) of which were represented in the SYT–SSX2-expressing C2C12 and hMSC microarrays (Supplementary Tables S1 and S2; synovial sarcoma tumors). A recent analysis of molecular targets that distinguished synovial sarcoma tumors (11 total) from non-sarcoma malignancies identified a similar array of key Wnt mediators and β-catenin targets (38; data not shown). Altogether, these gene profiling data indicate that an autocrine Wnt/β-catenin loop is active in human synovial sarcoma. Consistent with deregulated β-catenin as a major feature of synovial sarcoma in humans, we find that primary human synovial sarcoma tumors (4/4) display elevated levels of nuclear β-catenin (Supplementary Fig. S10A and S10B).

A striking feature of the SYT–SSX2-regulated Wnt/β-catenin targets (Supplementary Table S2) was their developmental nature. They included stem cell markers, embryonic lineage determinants, pluripotency factors, differentiation inhibitors, homeobox and forkhead box factors, as well as mediators of the fibroblast growth factor (FGF), NOTCH, Sonic hedgehog, and transforming growth factor β (TGFβ)/base morphogenetic protein (BMP) pathways. They all represent key regulators of embryonic development whose untimely upregulation in somatic tissues has been associated with human cancer (39–42). The embryonic nature of the SYT–SSX2 transcriptional targets led us to further search in the four gene arrays for mediators of tissue morphogenesis, known to regulate or be regulated by the Wnt/β-catenin pathway. This generated an extensive Wnt-centered network involving multiple embryonic lineages (Supplementary Fig. S11 and Supplementary Table S3) activated by SYT–SSX2 that appeared to persist in pleiomorphic sarcoma. The developmental nature of SYT–SSX2 gene targets was expected, as it is likely the result of SYT–SSX2 unique recruitment to Polycomb-silenced chromatin through the SSXR (10). In development, Polycomb stably represses lineage determinants and differentiation controllers to prevent untimely differentiation in stem cells (9). In summary, array analyses indicate that the Wnt/β-catenin cascade and the embryonic programs it controls appear to be active in SYT–SSX2-expressing mesenchymal stem/precursor cells, and in synovial sarcoma tumors.

DISCUSSION

In this report we show for the first time a constitutively active Wnt/β-catenin signal in SYT–SSX2-induced synovial sarcoma tumors that is necessary for synovial sarcoma initiation and growth. We show that targeting the aberrant Wnt/β-catenin signaling using small-molecule CK1α activators is capable of attenuating sarcoma growth. In addition, we present evidence that distinct SYT–SSX2 domains that exert epigenetic control over cellular differentiation overlap with domains that mediate Wnt/β-catenin pathway activity.
**Figure 7.** β-catenin nuclear signaling is mediated by SYT-SSX2 N-terminal residues and C-terminal SSXRD. A, schematic of SYT-SSX2 and its deletion mutants. SXd1SNH, SXd120, SXd140, SXd13, and SXd19 lack residues 35–55 and 65–78 of the SSXRD, respectively. Bottom panel, immunostaining of C2C12 myoblasts transduced with the designated retroviral vectors. POZ is empty vector. Expressed SYT-SSX2-derived proteins are HA (and FLAG)-tagged (green). POZ expresses an irrelevant small peptide that disappears with subcloning SYT-SSX2 and other cDNAs. β-catenin is visualized with a specific monoclonal antibody (red). The red and the green channels were merged (merge) for colocalization. Numbers represent average percent of HA-positive cells with nuclear β-catenin ± SD (n = 3). Five hundred nuclei were counted for each vector.

B, inset, FLAG immunoblot showing expression of SYT-SSX2 vectors. Histogram depicts fold increase of TOP-FLASH (T) activity with the SYT-SSX2-derived vectors relative to control vector (POZ). FOP-FLASH (F) was baseline activity; Error bars denote SDs; n = 3. P values were calculated relative to SYT-SSX2.
SYT–SSX2 and SYT–SSX1 are the most frequent types of SYT–SSX translocations in synovial sarcoma. Results from xenograft and in vitro studies, conducted with SYT–SSX1-expressing (HS-SY-II) and SYT–SSX2-expressing (mSS and SYO-1) synovial sarcoma cells indicates that our novel findings carry general significance for synovial sarcoma.

In uterine stroma, constitutive β-catenin expression is sufficient for development of mesenchymal tumors (43). Using synovial sarcoma xenografts and SYT–SSX2 transgenic models, we present evidence for a similar scenario. In the transgenic model where SYT–SSX2 expression in muscle precursors leads to synovial sarcoma tumor formation at 3 months of age with 100% penetrance, we show that such tumors fail to develop in a β-catenin–deficient background. Heterozygosity for β-catenin (SG2) led to a significant reduction in the number of detectable tumors, whereas visible tumors were absent in 3-month-old β-catenin–null SYT–SSX2 transgenic mice (SG3). The absence of detectable tumors in the latter strongly implicates the deregulation of β-catenin as a key feature in the initiation and development of synovial sarcoma tumors. This is further supported by SG3 outliers that have tumors displaying cells with strong nuclear β-catenin immunostaining, consistent with incomplete depletion by the Cre recombinase. These in vivo findings are consistent with our cellular data, where nuclear accumulation of β-catenin is detected within 48 hours of SYT–SSX2 transduction in the mesenchymal precursor cells. Because upregulation of Wnt/β-catenin signaling in cancer is often an indication of advanced malignant behavior, our studies support the notion of early acquisition of features of invasiveness conferred by the SYT–SSX fusion in the synovial sarcoma–initiating cell. The results provide a solid foundation to further examine Wnt involvement in the various stages of synovial sarcoma progression; namely initiation, local growth, and ultimately invasion and metastasis.

The inhibiting effects of pyrvinium and its analog, SSTC-104, on β-catenin in SYT–SSX2-expressing and mSS tumor cells indicated that CK1α activation is an effective mechanism to counteract β-catenin–mediated oncogenicity. Consistent with this, recent studies showed an anti-invasive function for CK1α (via inhibition of Wnt signaling) in malignant melanoma (44) and established CK1α as a potent negative regulator of the Wnt pathway in the intestine (45). Activating CTNNB1 mutations in human synovial sarcoma have been previously reported (20). CK1α agonists have the capacity to bypass key activating mutations (e.g., mutations in APC and β-catenin) of the Wnt pathway (29). Thus, we predict that SSTC-104 would be effective in synovial sarcoma tumors arising from activation of Wnt signaling due to genetic and epigenetic mechanisms.

The effects we observe with DKK1 indicate that the SYT–SSX2 oncogene generates receptor-mediated upstream signals to activate the Wnt/β-catenin cascade, and our microarray analyses revealed the upregulation of an autocrine Wnt/β-catenin loop by SYT–SSX2 expression. The current results point to proliferation as the primary Wnt activity at work in synovial sarcoma. One important phenotypic consequence shared by pyrvinium, SSTC-104, LRP6 knockdown, DKK1 treatment (data not shown), and SYT–SSX2 depletion in human synovial sarcoma cells (11) was a noticeable increase in cytoskeletal spread and formation of intercellular junctions enriched for β-catenin, indicating a common effect on relocating β-catenin to the cell surface. This characteristic deserves further investigation to clarify the molecular mechanism of the switch to a seemingly stronger adhesive state. Intriguingly, the effects of SSTC-104 were similar to those seen in β-catenin heterozygous mice (SG2), indicating that the concentration of SSTC-104 we used in our studies (3 mg/kg) is equivalent to removing one functional β-catenin allele. The SG1 mice provide an excellent model for testing drugs for treating synovial sarcoma, and our findings will serve as the basis for further studies where SSTC-104 and other Wnt inhibitors are explored for optimal doses and lengths of treatment.

In advanced human cancer, deregulation of Wnt/β-catenin signaling contributes to tumor progression. In several malignancies, it is rarely caused by Wnt-activating genetic mutations (e.g., malignant melanomas; ref. 46), and in certain cases, it occurs through epigenetic deregulation of its components (e.g., DKK3; refs. 47, 48). In the case of synovial sarcoma, our data is consistent with the latter, as activation of Wnt/β-catenin signaling appears to result from SYT–SSX function as a coregulator of gene expression, via its N-terminal and C-terminal (SSXRD) domains.

In embryogenesis, the Wnt pathway controls lineage allocation through orderly interactions with other signaling cascades (Supplementary Fig. S11). One such cascade is the FGF pathway previously shown to promote synovial sarcoma cell growth (6). Results of synovial sarcoma tumor formation in this SYT–SSX2/β-catenin model place Wnt/β-catenin signaling upstream of, or at a point of convergence with, the FGF cascade, in synovial sarcoma. Studies to clarify the mechanism of synergistic or additive interactions between the two pathways in synovial sarcoma will potentially benefit future synovial sarcoma therapies.

Upon SYT–SSX2 expression, and in synovial sarcoma tumors, it appears that the Wnt circuitry of lineage determinants is aberrantly mobilized (Supplementary Fig. S11 and Supplementary Table S3). Deregulation of embryonic determinants in adult somatic cells (6) could result in cellular catastrophe, as their normal function could transition the cell to a cancerous state (e.g., neuronal migration and gastrulation movements; Supplementary Fig. S11; ref. 6). The active Wnt/β-catenin cascade may therefore have far-reaching effects on the malignant state of the sarcoma. A key question concerning a direct collaboration between Wnt/β-catenin activation and SYT–SSX2 in transducing epigenetic control of gene expression remains to be addressed. SW1/SNF and Polycomb epigenetic effects are stably inherited; we therefore anticipate that SYT–SSX2, by functioning through both complexes, will maintain its dominant reprogramming and, consequently, an active Wnt/β-catenin signal, throughout the life of the tumor.

In summary, our current studies show that inappropriate activation of the Wnt/β-catenin pathway by the SYT–SSX oncogene is essential for sarcoma development in animal models. The prevalence of β-catenin deregulation in human synovial sarcoma tumors combined with our findings indicate that targeting Wnt in synovial sarcoma would benefit a substantial proportion of patients with synovial sarcoma.
METHODS

Cells
C2C12 cells were obtained from the American Type Culture Collection. MSCs were obtained from Dr. Prockop (Texas A&M) and Dr. Pamppe Young (Vanderbilt University, Nashville, TN). The SYO-1 and HS-SY-II cells were provided by Dr. Ladanyi (Memorial Sloan-Kettering Cancer Center, New York, NY). We authenticated all cell lines in differentiation assays and by conducting SYT–SSX RNAi and PCR testing (6, 2011). mSS cells were provided by Dr. Capezzi and authenticated by us (tumor xenografts) in the current study.

Mouse Procedures
Mice were acquired, housed, and bred following an approved protocol and rules set by the Institutional Animal Care and Use Committee at Vanderbilt University.

SSM2* and Myf5-Cre* transgenic mice were obtained from the Capezzi laboratory. The NUF/J nude and B-CATth/km mice were acquired from the Jackson Laboratory. The latter are B6.129-Ommrd1Cgrtm1Jkn (23). They carry two β-catenin alleles fixed between exons 2 and 6. Genotyping of all single, double, and triple mutant mice was conducted by PCR with primers recommended by the authors (22) and by the Jackson Lab. Synovial sarcoma tumors in 3-month-old SG1, SG2, and SG3 mice were detected and counted by internal and by the Jackson Lab. Synovial sarcoma tumors in 3-month-old SG1, SG2, and SG3 mice were detected and counted by internal inspection of the whole organism post-euthanasia. For tumors grown in nude mice, 2.5 × 10^6 mSS or HS-SY-II cells suspended in 100 μL PBS were implanted subcutaneously in the left hip area. Visible tumors were measured using a digital caliper.

Retroviral Infection and SYT–SSX2 Expression Vectors
Retroviral production and infection using the Phoenix packaging system, the double-tagged HA–FLAG–POZ template vector, POZ–SYT–SSX2–HA–FLAG, and POZ–SYT–HA–FLAG were described previously (11). Construction of POZ–SYT–SSX2–HA–FLAG deletion mutants designated SXdl1-d9 was described previously (7). Construction of the N-terminal SYT–SSX2 deletion mutants and LZRS-DKK1 is described in Supplementary Methods. Analyses were generally conducted 2 days postinfection. For sequential infections, LZRS-DKK1 was added 24 hours after POZ–SYT–SSX2 addition. DKK1 expression and β-catenin localization were quantified 48 hours after infection with LZRS-DKK1.

Immunohistochemical Procedures
Experimental details are described in the Supplementary Methods.

LRP6 Receptor Depletion by siRNA
LRP6 siRNA was conducted following manufacturer’s protocol (Dharmacon). A nontargeting (NT) and two targeting RNA oligomers, (NT): 5’-UAAGCUGUAUGAGAAGAUCAU-3’, si-LRP6-1: 5’-AGGAAUGUCCUGGAAAUAU-3’, and si-LRP6-2: 5’-GGAUAUGAAGGCAAU-3’ were added to cells. Cells were harvested for analysis 48 hours after transfection. The LRP6 protein was detected with an LRP6-specific rabbit monoclonal antibody (Cell Signaling Technology), and its relative intensities were measured using the FluorChem 8900 imaging system.

Luciferase Activity Assays
C2C12 or mSS cells were transfected using Superfect reagent (Qiagen), following manufacturer’s instructions. Luciferase activity was measured using the Promega Luciferase Reporter Assay Kit and a Pharmingen Monolight 3010 luminoimeter. In a total of 5 μg of transfected DNA, 0.5 μg of CMV-β-GAL was included as internal control. The DNA mix also contained 0.5 μg of LEF and 0.5 μg of β-catenin expression vectors, 0.5 μg of TOP-FFLASH or FOP-FFLASH (background control) reporter vector, and titrated amounts of SYT–SSX2, SXdl mutants, DKK1, or empty control vectors, depending on the experiment, except in the assays where mSS intrinsic Wnt/β-catenin activity is measured.

In vitro Treatment of Human SS Cells with SSTC-104
SYO-1 and HS-SY-II cells were plated in 6-well plates, in quadruplicate, at 1.5 × 10^4 cells per well. The following day, SSTC-104 dissolved in DMSO was added to the growth medium at 2 and 5 μmol/L final concentrations. A portion of SYO-1 cells was replenished with fresh medium containing SSTC-104 at 5 μmol/L and incubated for 2 additional days. At the end of the 2-day and 4-day SSTC-104 treatments, cells were measured for growth by manual count, and positivity for nuclear β-catenin was quantified. The relative intensities of de-phosphorylated β-catenin were measured using the FluorChem 8900 and the ImageJ imaging systems.

Treatment of Synovial Sarcoma Tumors with SSTC-104
2.5 × 10^6 HS-SY-II cells were implanted subcutaneously in the left flank. Four days later, tumors were measured with a digital caliper and SSTC-104 diluted in 15% DMSO/PBS was injected intraperitoneally at the designated doses. On the fourth day of treatment (day 8 of growth), the tumors were measured and a second dose of SSTC-104 was administered. Four days later (day 12 of growth), the tumors were measured and resected for analysis.

Microarray Data Mining
Details of the analyses are provided in Supplementary Methods.

Disclosure of Potential Conflicts of Interest
E. Lee is a Founder of StemSynergy Therapeutics. D. Orton has received a commercial research grant from StemSynergy Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: T.P. Sherrill, E. Lee, F. Yull, J.E. Eid Development of methodology: W. Barham, L. Gleaves, F. Yull, J.E. Eid Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Barham, A.L. Frump, T.P. Sherrill, C.B. Garcia, K. Saito-Diaz, L. Gleaves, D. Orton, M.R. Capecchi, T.S. Blackwell, J.E. Eid Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.B. Garcia, M.N. VanSaun, B. Fingleton, J.E. Eid Writing, review, and/or revision of the manuscript: W. Barham, K. Saito-Diaz, M.N. VanSaun, B. Fingleton, D. Orton, T.S. Blackwell, E. Lee, F. Yull, J.E. Eid Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.E. Eid Study supervision: F. Yull, J.E. Eid

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REFERENCES


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41. Rieger ME, Sims AH, Coats ER, Clarke RB, Briegel KJ. The embryonic transcription cofactor LBH is a direct target of the Wnt signaling pathway in epithelial development and in aggressive basal subtype breast cancers. Mol Cell Biol 2010;30:4267–79.


Targeting the Wnt Pathway in Synovial Sarcoma Models

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