Pharmacologic Inhibition of β-Catenin With Pyrvinium Inhibits Murine and Human Models of Wilms Tumor

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Wilms tumor (WT) is the most common renal malignancy in children and the fourth most common pediatric solid malignancy in the US. Although the mechanisms underlying the WT biology are complex, these tumors most often demonstrate activation of the canonical Wnt/β-catenin pathway. We and others have shown that constitutive activation of β-catenin restricted to the renal epithelium is sufficient to induce primitive renal epithelial tumors, which resemble human WT. Here we demonstrate that pharmacologic inhibition of β-catenin gene transcription with pyrvinium inhibits tumor growth and metastatic progression in a murine model of WT. Cellular invasion is significantly inhibited in both murine WT-like and human WT cells and is accompanied by downregulation of the oncogenes *Myc* and *Birc5* (survivin). Our studies provide proof of the concept that the canonical Wnt/β-catenin pathway may be a novel therapeutic target in the management of WT.

Key words: β-Catenin; Pyrvinium; Wilms tumor (WT)

INTRODUCTION

Wilms tumor (WT) is the most common renal malignancy in children[1,2]. Multimodal therapy, including cytotoxic chemotherapy, can cure the majority of children with WT, but at the cost of long-term toxicity[3–9]. Children who suffer from recurrent WT often do not respond to second-line therapy, and a significant proportion will die of their disease. Therefore, novel therapeutic approaches are needed to reduce the injurious effects of primary therapy while maintaining efficacy as well as improving outcomes among patients with recurrent disease. Identifying such novel targeted therapeutics requires a deeper understanding of the molecular pathways driving WT growth and progression.

WTs are triphasic, primitive renal epithelial malignancies that are thought to arise from primitive nephrogenic mesenchyme. The genetic alterations most commonly associated with WT include inactivating mutations of Wilms tumor 1 gene (*WT1*)[10,11], Wilms tumor gene found on chromosome X (*WTX*)[12–14], and stabilizing/activating mutations of β-catenin (*CTNNB1*)[15,16]. The precise mechanism by which these mutations lead to WT is not known, but they share the ability to activate the canonical β-catenin pathway[15,17–19].

The canonical β-catenin pathway plays a central role in the transcriptional regulation of a range of critical processes including cellular growth, differentiation, and development[20,21]. It has been implicated in the pathogenesis of several malignancies in addition to WT, including colon, breast, hepatocellular, and prostate cancers. Within the cytosol, β-catenin either associates with cadherin in the cellular membrane or exists in a free form. Normally, free β-catenin is tightly regulated by a degradation complex that includes Axin, WTX, GSK3β, and the APC protein. In the resting state, excess β-catenin is phosphorylated at its N terminus and thereby marked for

subsequent ubiquitination and proteasomal degradation. Wnt-related signaling interrupts this degradation, allowing β-catenin to accumulate within the cytosol, translocate to the nucleus, and there interact with the TCF/LEF family of transcription factors to promote gene expression[22].

In cancer, aberrant activation of the canonical β-catenin can occur through a variety of different mechanisms. The prototypical example is the loss of APC protein in familial adenomatosis polyposis leading to colon polyps and colon cancer. In sporadic WT, mutations in critical N-terminal serine/threonine residues that can prevent β-catenin's phosphorylation and degradation have been identified[15]. We and others have shown that such an activating mutation of β-catenin (*Ctnnb1*) is sufficient to induce renal epithelial tumors that resemble human WT[23–25]. We have shown that coordinate activation of Ras with β-catenin leads to rapidly progressive, metastatic tumors that demonstrate markedly increased canonical β-catenin pathway activation[23,24]. In a large series, similar activation has been demonstrated in the majority of human WT[26–28]. Collectively, this suggests that the canonical β-catenin pathway may represent a potential therapeutic target in WT, particularly in those patients with more advanced or high-risk disease.

Prior attempts to target the β-catenin pathway have been hampered by unacceptably high toxicity and poor tolerability. However, a well-tolerated oral agent, pyrvinium pamoate, which is approved for use as an antihelmintic, has recently been shown to inhibit β-catenin transcription through the activation of casein kinase 1α (CK1α)[29]. In preclinical models, pyrvinium has shown activity against other tumor types associated with canonical β-catenin pathway activation including breast cancer and intestinal polyposis[30,31]. Whether such an inhibitor of β-catenin signaling would be effective in the management of WT has not been tested.

We show that pyrvinium can block tumor growth and metastatic progression in a murine model of WT. Pharmacologic inhibition of β-catenin transcription abrogated Matrigel invasion in both murine WT-like (Kras/Catnb) and human WT (WiT49) cells in vitro while inhibiting cellular turnover in Kras/Catnb cells and migration and colony formation in WiT49 cells. This was associated with downregulation of the oncogenes *Myc* and *Birc5*, both target genes of canonical β-catenin activation that have been associated with the development and progression of human WT.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies used for immunohistochemistry (IHC) and/or Western blot were as follows: actin (Sigma-Aldrich, St Louis, MO, USA), Axin 2 and c-Myc (Abcam, Cambridge, MA, USA), FoxA2 (Santa Cruz Biotechnology,

Dallas, TX, USA), Ki-67 (DAKO/Agilent; Dako, Denmark), survivin and cleaved caspase 3 (Cell Signaling Technology, Boston, MA, USA). Pyrvinium was purchased from Sigma-Aldrich (#P0027).

Pyrvinium Treatment of Orthotopically Grafted WT Cells

To prepare the grafts, 10^5 Kras/Catnb cells were trypsinized, pelleted, and then resuspended in 60 μl of neutralized rat tail collagen as described previously[32]. Two collagen grafts were surgically placed in the sub-renal capsule of the left kidney of 7- to 8-week-old male athymic mice (Hsd:Athymic Nude-Foxn1nu; Harlan Laboratories, Indianapolis, IN, USA). The mice were allowed to recover for 14 days and then given a dose of 0.1 mg/kg of pyrvinium or control solution (DMSO and saline) via intraperitoneal (IP) injection. Mice were given gradually increased doses of 0.2 mg/kg at day 3, 0.4 mg/kg at day 6, 0.6 mg/kg at day 8, 0.8 mg/kg at day 10, and 1.0 mg/kg at day 13. Mice were then given the full dose of 1.0 mg/kg or control injections three times per week until the termination of the experiment. After a total treatment time of 3 weeks, mice were sacrificed, the kidneys and lungs were harvested, and both the grafted and contralateral nongrafted control kidneys were weighed. The tumor weight was expressed as the total weight of the grafted kidney normalized to its contralateral control as previously described[33]. Lung metastases were manually counted after hematoxylin and eosin (H&E) staining using three serial sections at two different depths within the lung tissue (six sections for each lung per mouse).

Histology and Immunohistochemistry

Murine kidneys were harvested, fixed in 10% buffered formalin, processed, and paraffin embedded. Sections were either stained with H&E or subjected to immunohistochemistry (IHC). For IHC, the slides were incubated with primary antibodies and then exposed to biotinylated secondary antibody followed by incubation with an ABC-HRP complex (Vector Laboratories, Burlingame, CA, USA) and then with liquid 3,3′-diaminobenzidine tetrahydrochloride (DAB; DAKO liquid DAB + substrate chromogen system; Carpinteria, CA, USA). Stained sections were photographed and processed using a Zeiss AX10 Imager.M1 microscope and AxioVision Release 4.6 software.

Cell Viability Assay

Cell viability was determined using the MTS method (Promega, Madison, WI, USA) using the manufacturer's protocol. In brief, cells were seeded into 96-well culture plates, grown overnight, and treated as indicated. MTS/PMS solution was added for 1 h, and absorbance at 490 nm was measured. All experiments were completed

in triplicate, and the results were provided as the mean± the standard error (SE).

Tritiated Thymidine Incorporation

Cells were seeded onto 35-mm dishes and treated as indicated. Tritiated thymidine was added, and the cells were incubated for 2 h. The medium was removed, and the cells were incubated with 10% TCA solution, washed twice, and incubated with 0.2 N NaOH. Aliquots were combined with scintillation fluid and counted on a scintillation counter. All experiments were completed in triplicate.

Cellular Migration/Wound Healing Assay

Cells were grown in six-well plates to 100% confluence and pretreated with reduced serum (1% FBS) medium overnight. Medium was removed, and several parallel scratch lines (wounds) were made with a sterile 200-μl pipette tip. Dislodged cells and debris were gently removed by washing with PBS and serum-reduced media with or without inhibitors added. Baseline images of the same spots (at least four per well) were captured immediately and 6, 16, 24, and 48 h after scratching. The distance between wound borders was measured using cellSens software (Olympus Corp., Tokyo, Japan) as the average of 15 parallel lines connecting cells across the wound. The average±SE difference was then calculated. Each experiment was repeated in triplicate.

Cellular Invasion Assay

BD BioCoat™ Matrigel™ Invasion chambers (Cat. No. 354480; BD Biosciences, Bedford, MA, USA) were utilized according to the manufacturer's protocol. After warming and rehydration, 10^5 cells were seeded with inhibitors or vehicle in serum-free cell culture medium in the upper chamber/inserts, and full serum medium with the same inhibitor was placed into the lower chamber/wells and incubated for 24 h. Inserts were then removed and fixed in 10% neutral-buffered formalin and stained with Mayer's hematoxylin. Cells on the inner aspect of the insert were removed with a cotton swab, while cells on the outer membrane were counted by cutting out the insert and mounting them on a slide with a coverslip and allowed to dry overnight. Ten nonoverlapping pictures were captured for each membrane using cellSens Life Science Imaging Software (Olympus Corp.). Cells were then counted using the same software, and the mean±SE was calculated. Each experiment was repeated in triplicate.

Colony Formation in Soft Agar

Six-well plates were coated with a 1:1 mix of 1.6% sea plaque agarose (Cambrex Bio Science, Rockland, ME, USA) and 2× cell culture medium (with all additives and 2× serum) and allowed to solidify. A mix of 2× cell culture medium, sea plaque agarose, and 5,000 cells with inhibitors or vehicle in 1× cell culture medium (ratio 1:1:2 by volume) was plated above the soft agar coat, allowed to solidify, and incubated at 37°C for 4 weeks. The total number, size, and density of colonies were captured using the GelCount™ system (Oxford Optronix, Abingdon, UK) that includes the digital image capture and analysis software. Each experiment was repeated three times with representative results shown.

Immunoblotting

Cells were washed and dissolved in lysis buffer [made fresh from a 6× stock solution of 2 M Tris-HCl (pH 6.8), 20% SDS, glycerol, and protease inhibitors] and sonicated. Cell lysates were cleared by centrifugation. Protein concentration was determined using the Bio-Rad protein assay and then subjected to SDS-PAGE, transferred to Immobilon-P transfer membranes (Millipore Corp., Boston, MA, USA), and subjected to immunoblot analysis utilizing standard methods using the antibodies listed previously.

RT-PCR

cDNA was synthesized using Superscript reverse transcription (RT) reagents (Life Technologies, Grand Island, NY, USA). Real-time PCR was performed using Lumino Ct® SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich) and CFX96 Touch Real-Time System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The mouse primers were as follows: *Gapdh*, 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-CACCACCCTGTTGCTGTAGCC-3′ (reverse) (100 ng of cDNA); *Axin2*, 5′-GGACAGTAGCGTAGATGGAG-3′ (forward) and 5′-CGGAAAATGAGGTAGAGACA-3′ (reverse) (100 ng of cDNA): *Foxa2*, 5′-AGCACCAT TACGCCTTCAAC-3′ (forward) and 5′- CCTTGAGG TCCATTTTGTGG-3′ (reverse) (50 ng of cDNA); *Myc*, 5′-CGACTACGACTCCGTACAGC-3′ (forward) and 5′-GTAGCGACCGCAACATAGGA-3′ (reverse) (50 ng of cDNA); and *Birc5*, 5′-ACCGAGAACGAGCCTGA TTT-3′ (forward) and 5′-GCTGCTCAATTGACTGA CGG-3′ (reverse) (50 ng of cDNA). For each reaction, the annealing temperature was 65°C using the indicated amount of cDNA per reaction. Comparisons of expression by RT-PCR across groups were done using the $2^{-\Delta\Delta CT}$ method[34]. Analysis was completed using GraphPad Prism v5.02 (La Jolla, CA, USA).

Statistical Analysis

Descriptive statistics were expressed as the mean±SE. Proportional differences were compared using contingency tables and Fisher's exact test. Comparisons of continuous variables were done with Mann–Whitney test or one-way ANOVA and Kruskal–Wallis test. All tests were completed using PRISM 5.0d© (GraphPad Software Inc.).

RESULTS

Inhibition of β-Catenin Transcription Decreases Tumor Growth and Metastatic Progression in a Murine Model of WT

To test whether pharmacologic inhibition of β-catenin transcription can inhibit growth and metastatic disease progression, we grafted murine renal epithelial cells with activating mutations of both Kras and β-catenin (Kras/Catnb cells) under the renal capsule of nude mice as previously described[33]. We have previously shown that when grafted orthotopically, these cells grow into tumors, metastasize to the lung, and recapitulate many of the features of human WT[33]. Compared to placebo-treated control, 4 weeks of pyrvinium treatment significantly reduced the size of the primary tumors (Fig. 1). It also profoundly reduced the number and size of the lung metastases seen at necropsy (Fig. 2). This was due to inhibition of tumor growth as assessed by Ki-67 staining (Fig. 3A and B). However, there was no appreciable difference in apoptotic cell death as assessed by cleaved caspase 3 staining (Fig. 3C and D). Importantly, pyrvinium treatment was well tolerated, and no mice demonstrated signs of toxicity due to the therapy.

Pyrvinium Significantly Reduces Cell Growth and Invasion While Modestly Decreasing Cellular Migration and Colony Formation in a Murine Model of WT

To test which cellular processes are modulated by the canonical β-catenin pathway to drive tumor growth and metastatic disease progression, we assessed the effect of pyrvinium on Kras/Catnb cells in vitro. Compared to the control, pharmacologic inhibition of β-catenin transcription with pyrvinium significantly reduced the growth kinetics of Kras/Catnb cells over time as assessed by the MTS assay (Fig. 4A). This was accompanied by profound inhibition of cell proliferation as measured by tritiated thymidine incorporation (Fig. 4B). Both cellular migration and invasion are important measures of a tumor cell's metastatic potential. Inhibition of β-catenin transcription had a profound dose-dependent effect on cellular invasion when assessed by Transwell invasion assay (Fig. 4D)
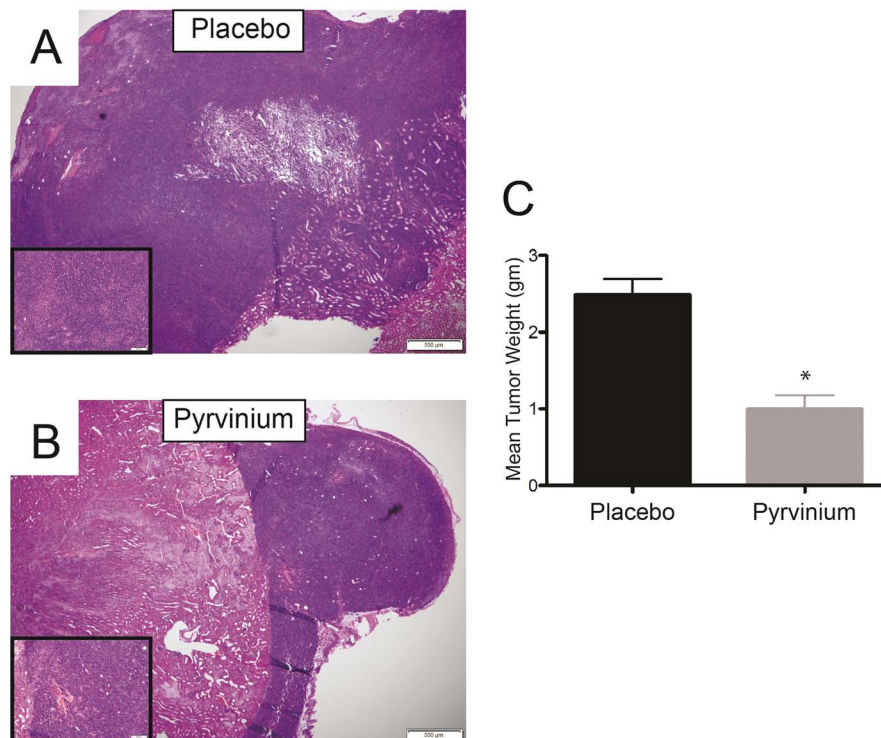
Figure 1. Pyrvinium decreases tumor growth and metastatic progression in a murine model of Wilms tumor (WT). Murine Kras/Catnb cells were orthotopically grafted into the subrenal capsule of nude mice and allowed to grow for 2 weeks. Mice were treated for 3 weeks with intraperitoneal (IP) injections of pyrvinium or vehicle control. Kidneys and lungs were harvested, weighed, and fixed in formalin. Hematoxylin and eosin (H&E) staining showed significantly larger tumors in vehicle-treated controls (A) compared to pyrvinium-treated (B) animals (image shown is 4×, scale bar: 500 μm; inset shows 20× image, scale bar: 100 μm). (C) Tumor weight was calculated by weighing the tumor-bearing kidney normalized by the contralateral control/nongrafted kidney from the same mouse. Pyrvinium-treated mice ($n = 11$) had significantly smaller tumors (median: 0.998 g) compared to vehicle control ($n = 12$, mean: 2.488 g). *$p = 0.0005$.
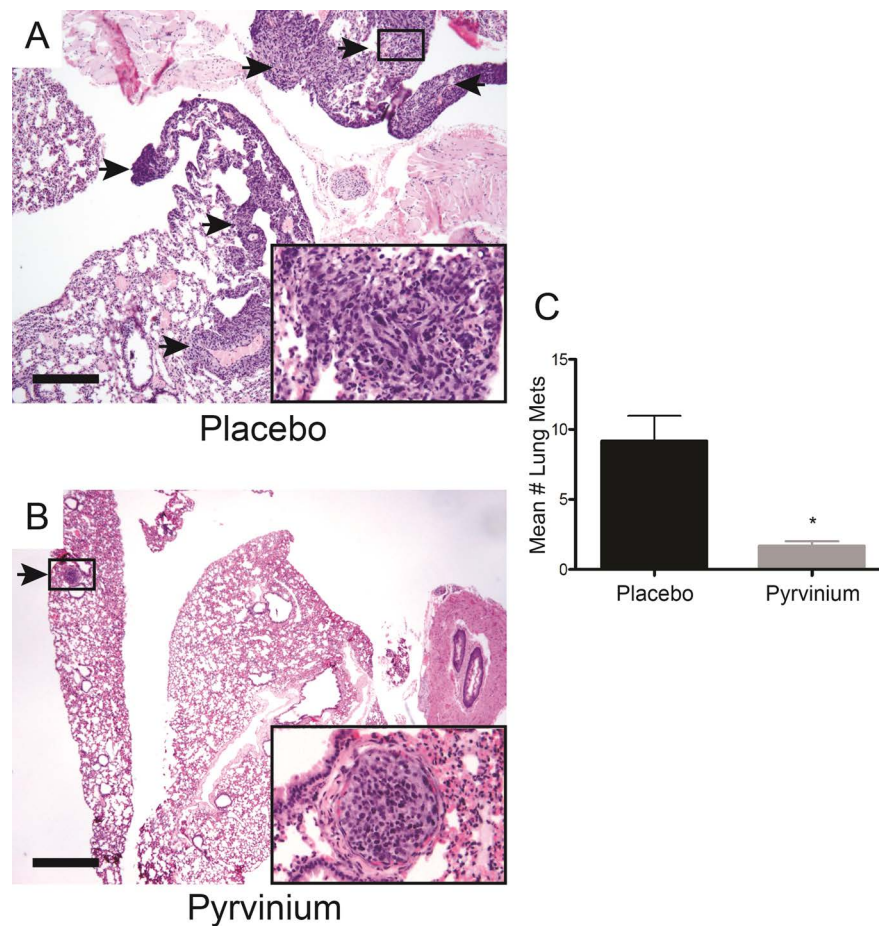
Figure 2. Pyrvinium decreases lung metastases from Kras/Catnb orthotopic tumors. Mice bearing orthotopic subrenal grafts of Kras/Catnb cells were treated with pyrvinium or vehicle control for 3 weeks, and the lungs were harvested, fixed, and H&E stained. There were fewer lung metastases (arrows) in pyrvinium-treated mice (B) compared to vehicle control (A). The image shown is 4×, scale bar: 500 μm; inset shows 20× image of the area outlined. The mean number of metastases (C) was significantly lower in the pyrvinium-treated mice (mean: 1.67, SD: 1.23 in 11 mice) compared to vehicle control (mean: 9.18, SD: 5.91 in 12 mice). *$p < 0.0001$.

but only modestly affected cellular migration (Fig. 4C). Colony formation in soft agar by Kras/Catnb cells was also moderately affected by pyrvinium (Fig. 4E). Taken together, this suggests that pharmacologic inhibition of β-catenin transcription significantly inhibits Kras/Catnb cellular proliferation and invasion while also affecting migration and colony formation in soft agar.

Inhibition of β-Catenin Transcription With Pyrvinium Decreases Expression of Myc and Birc

To define candidate molecular pathways that may drive metastatic potential in murine WT-like cells, we examined the protein expression of canonical β-catenin target genes inhibited by pyrvinium in Kras/Catnb cells. *Axin2* and *Foxa2* are both prototypical downstream targets of Wnt/β-catenin, and their expressions were both inhibited by pyrvinium, thus confirming inhibition of the canonical Wnt/β-catenin pathway (Fig. 5A). Interestingly,

the protein level of oncogenes *Myc* and *Birc5* were also reduced by exposure to pyrvinium (Fig. 5A). We confirmed that mRNA expression was inhibited through RT-PCR of *Axin2, Foxa1, Myc,* and *Birc5* in pyrvinium-treated Kras/Catnb cells relative to the control (Fig. 5B). This suggests that pyrvinium treatment, which decreases cellular oncogenic potential, inhibits β-catenin transcription and downregulates potentially critical β-catenin-targeted oncogenes.

Pyrvinium Impairs Human WT Cellular Invasion, Migration, and Colony Formation and Is Associated With Reduced Myc and Survivin Expression

To confirm these observations in a human cell line, we tested pyrvinium's effect on the WT cell line WiT49. Similar to Kras/Catnb cells, pharmacologic inhibition of β-catenin profoundly reduced cellular invasion (Fig. 6D) in a dose-dependent fashion. There was also significant
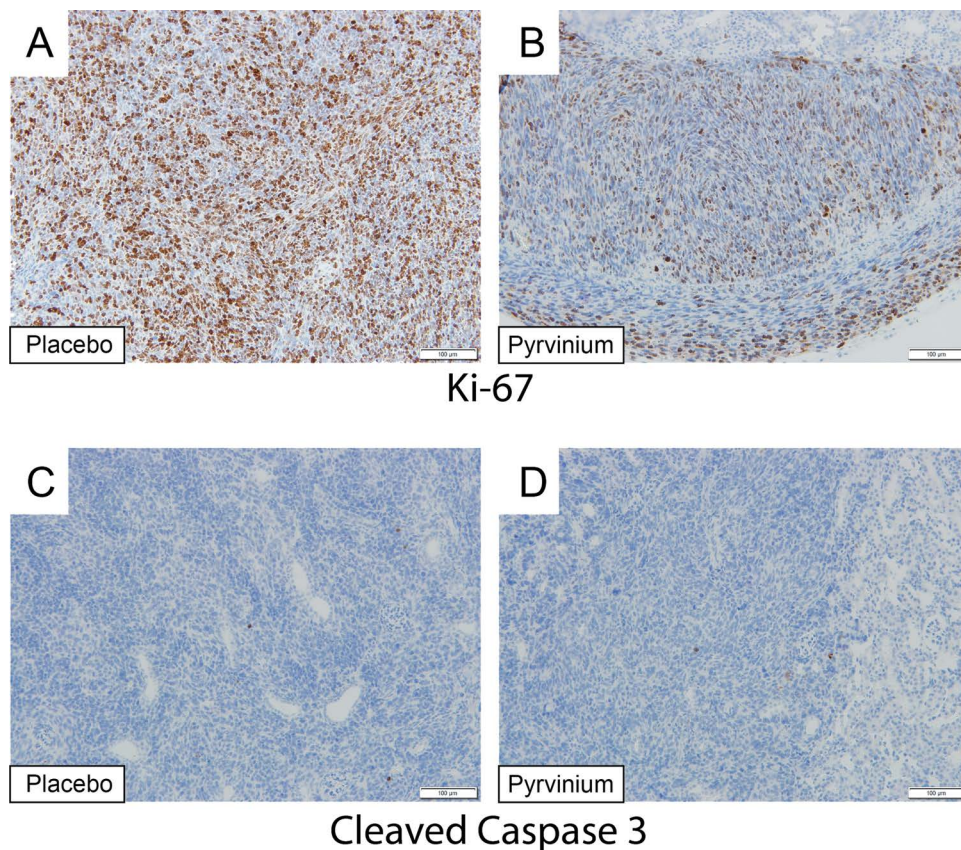
Figure 3. Pyrvinium abrogates cellular growth but not apoptosis of Kras/Catnb orthotopic grafts. Orthotopic subrenal capsular tumors from Kras/Catnb cells treated with pyrvinium or vehicle control were harvested, fixed, and subjected to immunohistochemistry (IHC). Tumors from pyrvinium-treated mice had less growth by staining for Ki-67 (B) than vehicle-treated controls (A). The levels of apoptosis assessed by IHC for cleaved caspase 3 were low for both pyrvinium-treated (D) and vehicle control-treated (C) mice. Images shown are 20×, scale bar: 100 μm.

inhibition of cellular migration (Fig. 6C) and colony formation in soft agar (Fig. 6E). There was less effect on cellular growth kinetics (Fig. 6A) and no appreciable effect on cellular proliferation (Fig. 6B). As with Kras/Catnb, protein expression of the canonical Wnt/β-catenin target genes *Axin2* and *Foxa2* was significantly inhibited by pyrvinium in human Wit49 cells as well as the oncogenes *Myc* and *Birc5* (survivin).

DISCUSSION

The canonical β-catenin pathway is activated in the majority of human WT[26–28], and an activating mutation is sufficient to induce WT in mice[23–25]. Tumor growth and metastatic progression are accelerated by coordinate activation of Ras, which is associated with increased β-catenin activation[23]. This suggests that β-catenin plays an important role in WT progression. Here we show that a well-tolerated oral inhibitor of canonical β-catenin transcription, pyrvinium, can inhibit growth and metastatic disease progression in a murine model of WT. We show that pyrvinium inhibits invasion of both murine WT-like

and human WT cells in vitro and downregulates the *Myc* and *Birc5* oncogenes in a dose-dependent fashion. This suggests that inhibition of β-catenin transcription could be a useful therapeutic target in the management of human WT.

Pyrvinium is a quinoline-derived cyanine dye that was originally approved for use in the 1950s for the management of enteric infections with the pinworm *Enterobius vermicularis*[35]. More recently, studies have suggested that it may be a useful antineoplastic agent that has an inhibitory effect in pancreatic and colon cancers as well as synovial sarcoma models[36–38]. It has been shown to synergize with cytotoxic chemotherapeutic agents such as doxorubicin and 5-FU to induce apoptosis in tumor cell lines[29,37]. The antineoplastic effect in colon cancer cells was shown to be due, at least in part, to its ability to inhibit canonical Wnt/β-catenin activation through the activation of CK1α, although it should be acknowledged that pyrvinium could have additional off-target effects[29]. Although CK1α is involved in the phosphorylation and degradation of β-catenin in the cytosol, in the nucleus it
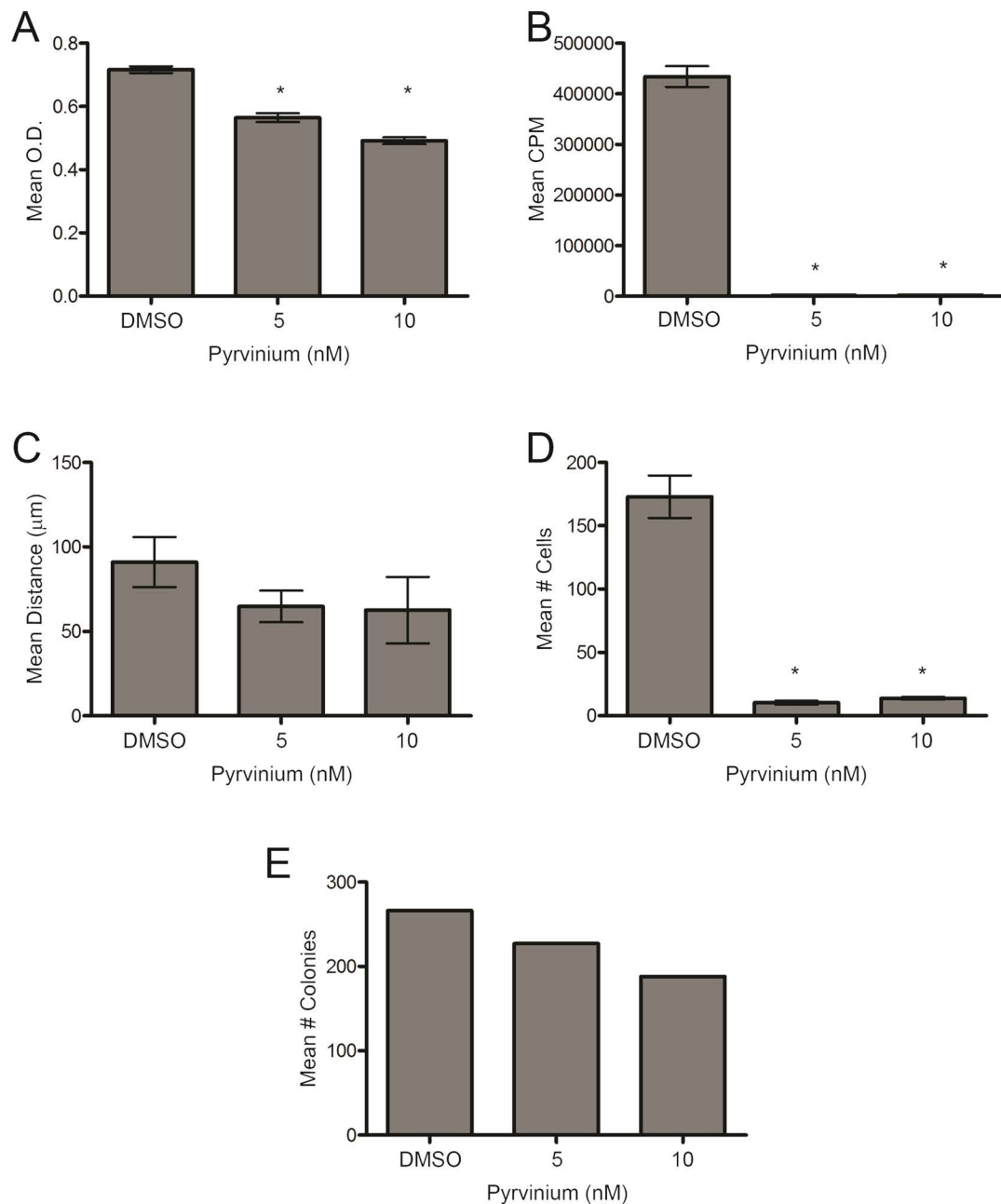
Figure 4. Pyrvinium significantly reduced cell growth and invasion in a murine model of WT. Kras/Catnb cells treated with pyrvinium showed significant inhibition of cellular kinetics by MTS assay (A) and cell proliferation by tritiated thymidine incorporation (B) when compared to control. There was also significant, dose-dependent inhibition of cellular invasion (D) and a more modest effect on migration (C) and colony formation in soft agar (E). All experiments were done in 1% FBS, repeated in triplicate, and representative results are shown. *$p < 0.05$.

also acts to lower levels of Pygopus, an important transcriptional regulator of β-catenin. Pyrvinium was shown to inhibit Wnt signaling using an assay that measures secondary axis formation of *Xenopus* embryos and, consistent with the current study in WT cells, expression of the Myc oncogene in colorectal cancer cells[29]. While we have not formally tested that these mechanisms are identical in our model system, we also observed downregulation of the canonical Wnt/β-catenin target genes *Axin2*, *Foxa2*, *Birc5*, and *Myc*.

Collectively, these studies present proof of the concept that drugs such as pyrvinium might be novel antineoplastic agents, either on their own or in combination with cytotoxic chemotherapeutics. Indeed, several studies
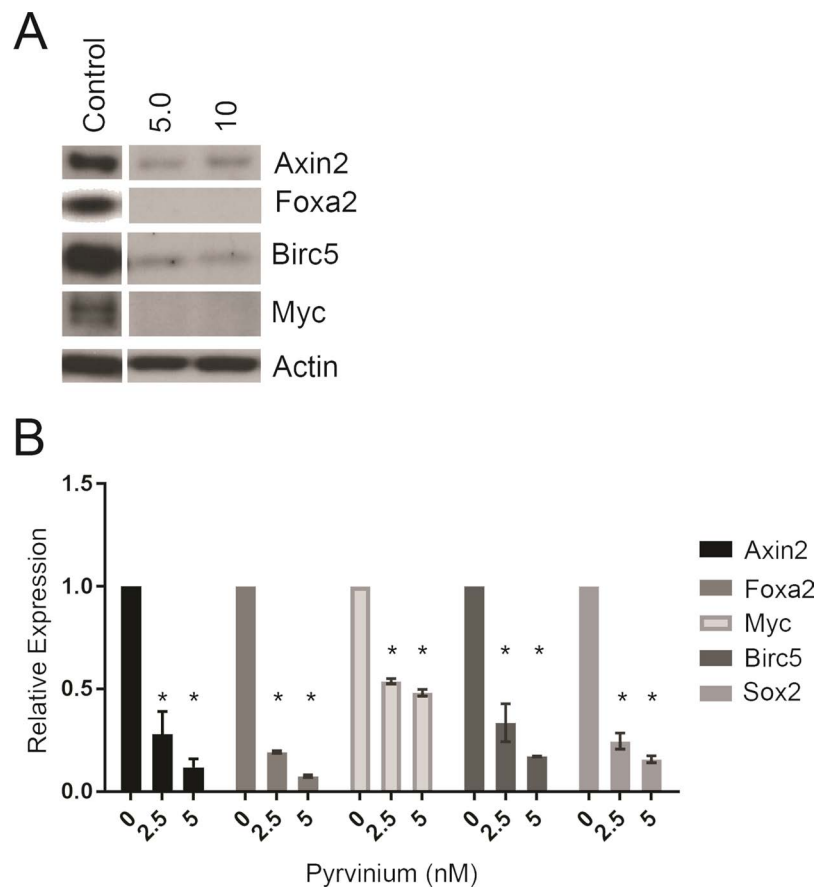
Figure 5. Pyrvinium inhibits expression of the β-catenin target genes *Axin2*, *Foxa2*, *Myc*, and *Birc5* in Kras/Catnb cells. Immunoblot shows decreased *Axin2*, *FoxA2*, *c-Myc*, and survivin (*Birc5*) in cells maintained in 1% FBS and treated with pyrvinium (A). Decreased gene transcription was confirmed by reverse transcription polymerase chain reaction (RT-PCR) normalized to expression of *Gapdh* (B). All experiments were done in triplicate with representative results shown. $*p < 0.05$.

have suggested that Wnt inhibition might be useful in other contexts such as orthopedic injuries or cardiovascular disease[39–41]. However, the poor oral bioavailability of pyrvinium, while completely appropriate to treat enteric helminthic infections, represents a significant barrier to its use in patients for other purposes. Our studies used an IP injection of pyrvinium in mice, but such alternative routes of administration in humans have not been extensively studied. In an effort to overcome these barriers, there have been small-molecule screens that have suggested that other potential inhibitors of β-catenin pathway exist and can be effective[42–44]. For example, PRI-724 is an antagonist of canonical β-catenin transcription that inhibits the interaction between β-catenin and cAMP response element-binding protein (CREB)-binding protein (CBP). It has been shown to be tolerated in human phase I studies with planned phase II efficacy studies in combination with the anti-VEGF antibody bevacizumab planned for patients with metastatic colorectal carcinoma[42,44]. Another

small-molecule inhibitor, CWP232291, which binds to Src-associated substrate in mitosis of 68 kDa (Sam68), has been found to inhibit canonical β-catenin transcription and is being tested in a phase I toxicity trial for patients with hematologic malignancies[42,43]. Whether such agents have a role in WT or other malignancies remains to be determined, but our studies offer proof of the principle that such investigations are warranted.

We found that pyrvinium consistently decreased cellular invasion in vitro in both murine and human WT cell lines and could also inhibit cellular migration, proliferation, and colony formation. This is consistent with studies showing that the canonical Wnt/β-catenin pathway plays an important role in cellular migration and invasion in multiple tumor types including breast, prostate, gastric, hepatocellular, lung, and thyroid carcinoma cells[45–51]. Pyrvinium has been shown to inhibit β-catenin-driven gene transcription, but multiple different mechanisms can inhibit the Wnt/β-catenin pathway including interruption
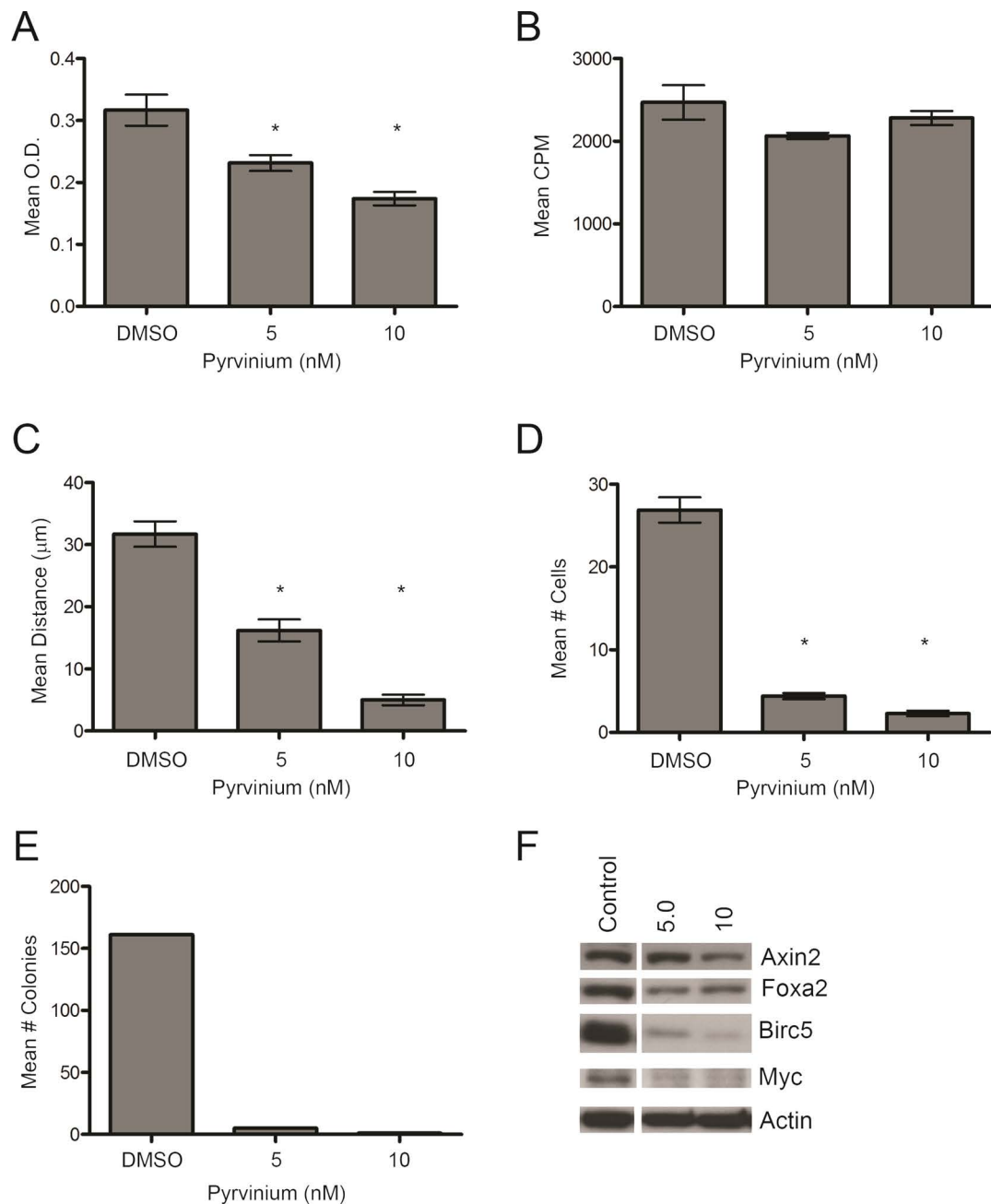
Figure 6. Pyrvinium significantly reduces cell migration, invasion, and colony formation in human WiT49 cells. Treatment with pyrvinium significantly reduced cellular migration (C), invasion (D), and colony formation (E) of human WT cells (WiT49). Cellular growth by MTS assay (A) was only modestly affected, while there was no appreciable effect on cell proliferation as measured by tritiated thymidine incorporation (B). Immunoblot showed decreased expression of *Axin2*, *FoxA2*, *c-Myc*, and survivin in WiT49 cells after pyrvinium exposure (F). All experiments were done in triplicate with representative results shown. *$p < 0.05$.

of Wnt interaction with the Frizzled/LRP receptor[45,46,50], decreasing Wnt ligands[49], abrogating β-catenin degradation[51], or altering β-catenin's interaction with transcription factors in the nucleus affecting gene transcription[48]. This suggests that drug targeting could be tested at a variety of different points in the Wnt/β-catenin signaling cascade, making this a fruitful future direction in cancer therapy, including in WT.

We show that treatment of murine and human WT cells with pyrvinium downregulates both *Myc* and *Birc5*[52,53].

Myc is a downstream target of activated β-catenin signaling that has been implicated in malignant transformation due to its role in regulating gene transcription[54]. We have shown that a gene expression signature that distinguishes a mouse model and human WT from normal human kidney or other neoplasms includes marked upregulation of c-Myc[24]. Mutations in the YEATS domain of the *MLLT1* gene have recently been linked to the development of WT and are characterized by increased *Myc* expression[55]. Prior work has shown that the kidneys of mice harboring activating mutations of K-Ras and β-catenin restricted to the renal epithelium develop metastatic WT and have significantly increased c-Myc and *Birc5*'s protein product, survivin[24]. Survivin is a member of the inhibitor of apoptosis (IAP) family of proteins and, like c-Myc, a downstream target of the canonical Wnt/β-catenin pathway[53]. Through its role in inhibiting cellular apoptosis, it has been associated with human malignancy and is being investigated as a potential therapeutic target[56]. *Birc5* expression is markedly higher in WT when compared to normal kidneys[57] and has been associated with WT disease stage[58], and polymorphisms in the gene are associated with WT risk[59]. A selective survivin inhibitor, YM155, has also been shown to induce apoptosis in a post-chemotherapy-derived WT cell line (SK-NEP-1)[60]. Collectively, this suggests that both *Myc* and *Birc5* (survivin) play an important role in WT, and one mechanism by which pyrvinium inhibits WT growth and metastases could be through their downregulation. Confirmatory studies are required to test this hypothesis.

In conclusion, we have shown that pharmacologic inhibition of the canonical Wnt/β-catenin pathway with pyrvinium can inhibit growth and metastatic progression of murine and human cellular models of WT and is associated with marked downregulation of *Myc* and *Birc5*. Our study demonstrates the potential for targeting the canonical Wnt/β-catenin pathway in the management of WT.

REFERENCES

1. Grovas A, Fremgen A, Rauck A, Ruymann FB, Hutchinson CL, Winchester DP, Menck HR. The National Cancer Data Base report on patterns of childhood cancers in the United States. Cancer 1997;80(12):2321–32.

2. Gurney JG, Severson RK, Davis S, Robison LL. Incidence of cancer in children in the United States. Sex-, race-, and 1-year age-specific rates by histologic type. Cancer 1995; 75(8):2186–95.

3. Dome JS, Cotton CA, Perlman EJ, Breslow NE, Kalapurakal JA, Ritchey ML, Grundy PE, Malogolowkin M, Beckwith JB, Shamberger RC, Haase GM, Coppes MJ, Coccia P, Kletzel M, Weetman RM, Donaldson M, Macklis RM, Green DM. Treatment of anaplastic histology Wilms' tumor: Results from the fifth National Wilms' Tumor Study. J Clin Oncol. 2006;24(15):2352–8.

4. Metzger ML, Dome JS. Current therapy for Wilms' tumor. Oncologist 2005;10(10):815–26.

5. Sonn G, Shortliffe LM. Management of Wilms tumor: Current standard of care. Nat Clin Pract Urol. 2008; 5(10):551–60.

6. Green DM, Grigoriev YA, Nan B, Takashima JR, Norkool PA, D'Angio GJ, Breslow NE. Congestive heart failure after treatment for Wilms' tumor: A report from the National Wilms' Tumor Study group. J Clin Oncol. 2001;19(7):1926–34.

7. Green DM, Peabody EM, Nan B, Peterson S, Kalapurakal JA, Breslow NE. Pregnancy outcome after treatment for Wilms tumor: A report from the National Wilms Tumor Study Group. J Clin Oncol. 2002;20(10):2506–13.

8. Jones DP, Spunt SL, Green D, Springate JE. Renal late effects in patients treated for cancer in childhood: A report from the Children's Oncology Group. Pediatr Blood Cancer 2008;51(6):724–31.

9. Taylor AJ, Winter DL, Pritchard-Jones K, Stiller CA, Frobisher C, Lancashire ER, Reulen RC, Hawkins MM. Second primary neoplasms in survivors of Wilms' tumour—A population-based cohort study from the British Childhood Cancer Survivor Study. Int J Cancer 2008; 122(9):2085–93.

10. Huff V. Wilms tumor genetics. Am J Med Genet. 1998; 79(4):260–7.

11. Ruteshouser EC, Robinson SM, Huff V. Wilms tumor genetics: Mutations in WT1, WTX, and CTNNB1 account for only about one-third of tumors. Genes Chromosomes Cancer 2008;47(6):461–70.

12. Rivera MN, Kim WJ, Wells J, Driscoll DR, Brannigan BW, Han M, Kim JC, Feinberg AP, Gerald WL, Vargas SO, Chin L, Iafrate AJ, Bell DW, Haber DA. An X chromosome gene, WTX, is commonly inactivated in Wilms tumor. Science 2007;315(5812):642–5.

13. Perotti D, Gamba B, Sardella M, Spreafico F, Terenziani M, Collini P, Pession A, Nantron M, Fossati-Bellani F, Radice P. Functional inactivation of the WTX gene is not a frequent event in Wilms' tumors. Oncogene 2008;27(33):4625–32.

14. Fukuzawa R, Holman SK, Chow CW, Savarirayan R, Reeve AE, Robertson SP. WTX mutations can occur both early and late in the pathogenesis of Wilms tumour. J Med Genet. 2010;47(11):791–4.

15. Koesters R, Ridder R, Kopp-Schneider A, Betts D, Adams V, Niggli F, Briner J, von Knebel Doeberitz M. Mutational activation of the beta-catenin proto-oncogene is a common event in the development of Wilms' tumors. Cancer Res. 1999;59(16):3880–2.

16. Maiti S, Alam R, Amos CI, Huff V. Frequent association of beta-catenin and WT1 mutations in Wilms tumors. Cancer Res. 2000;60(22):6288–92.

17. Kim MK, McGarry TJ, P OB, Flatow JM, Golden AA, Licht JD. An integrated genome screen identifies the Wnt signaling pathway as a major target of WT1. Proc Natl Acad Sci USA 2009;106(27):11154–9.

18. Kim MS, Yoon SK, Bollig F, Kitagaki J, Hur W, Whye NJ, Wu YP, Rivera MN, Park JY, Kim HS, Malik K, Bell DW, Englert C, Perantoni AO, Lee SB. A novel Wilms tumor 1 (WT1) target gene negatively regulates the WNT signaling pathway. J Biol Chem. 2010;285(19):14585–93.

19. Major MB, Camp ND, Berndt JD, Yi X, Goldenberg SJ, Hubbert C, Biechele TL, Gingras AC, Zheng N, Maccoss MJ, Angers S, Moon RT. Wilms tumor suppressor WTX negatively regulates WNT/beta-catenin signaling. Science 2007;316(5827):1043–6.

20. Clevers H. Wnt/beta-catenin signaling in development and disease. Cell 2006;127(3):469–80.

21. Klaus A, Birchmeier W. Wnt signalling and its impact on development and cancer. Nat Rev Cancer 2008;8(5): 387–98.

22. Saito-Diaz K, Chen TW, Wang X, Thorne CA, Wallace HA, Page-McCaw A, Lee E. The way Wnt works: Components and mechanism. Growth Factors 2013;31(1):1–31.

23. Clark PE, Polosukhina D, Love H, Correa H, Coffin C, Perlman EJ, de Caestecker M, Moses HL, Zent R. β-Catenin and K-RAS synergize to form primitive renal epithelial tumors with features of epithelial Wilms' tumors. Am J Pathol. 2011;179(6):3045–55.

24. Yi Y, Polosukhina D, Love HD, Hembd A, Pickup M, Moses HL, Lovvorn HN, 3rd, Zent R, Clark PE. A murine model of K-RAS and beta-catenin induced renal tumors expresses high levels of E2F1 and resembles human Wilms tumor. J Urol. 2015;194(6):1762–70.

25. Huang L, Mokkapati S, Hu Q, Ruteshouser EC, Hicks MJ, Huff V. Nephron progenitor but not stromal progenitor cells give rise to Wilms tumors in mouse models with beta-catenin activation or Wt1 ablation and Igf2 upregulation. Neoplasia 2016;18(2):71–81.

26. Koesters R, Niggli F, von Knebel Doeberitz M, Stallmach T. Nuclear accumulation of beta-catenin protein in Wilms' tumours. J Pathol. 2003;199(1):68–76.

27. Li CM, Kim CE, Margolin AA, Guo M, Zhu J, Mason JM, Hensle TW, Murty VV, Grundy PE, Fearon ER, D'Agati V, Licht JD, Tycko B. CTNNB1 mutations and over-expression of Wnt/beta-catenin target genes in WT1-mutant Wilms' tumors. Am J Pathol. 2004;165(6):1943–53.

28. Su MC, Huang WC, Lien HC. Beta-catenin expression and mutation in adult and pediatric Wilms' tumors. APMIS 2008;116(9):771–8.

29. Thorne CA, Hanson AJ, Schneider J, Tahinci E, Orton D, Cselenyi CS, Jernigan KK, Meyers KC, Hang BI, Waterson AG, Kim K, Melancon B, Ghidu VP, Sulikowski GA, LaFleur B, Salic A, Lee LA, Miller DM 3rd, Lee E. Small-molecule inhibition of Wnt signaling through activation of casein kinase 1alpha. Nat Chem Biol. 2010;6(11):829–36.

30. Li B, Flaveny CA, Giambelli C, Fei DL, Han L, Hang BI, Bai F, Pei XH, Nose V, Burlingame O, Capobianco AJ, Orton D, Lee E, Robbins DJ. Repurposing the FDA-approved pin-worm drug pyrvinium as a novel chemotherapeutic agent for intestinal polyposis. PLoS One 2014;9(7):e101969.

31. Xu W, Lacerda L, Debeb BG, Atkinson RL, Solley TN, Li L, Orton D, McMurray JS, Hang BI, Lee E, Klopp AH, Ueno NT, Reuben JM, Krishnamurthy S, Woodward WA. The antihelmintic drug pyrvinium pamoate targets aggressive breast cancer. PLoS One 2013;8(8):e71508.

32. Hallowes RC, Bone EJ, Jones W. A new dimension in the culture of human breast. In: Rajan RJRaKT, editor. Tissue culture in medical research. Volume 1. Oxford: Pergamon Press; 1980. p 213–20.

33. Polosukhina D, Love HD, Correa H, Su Z, Dahlman KB, Pao W, Moses HL, Arteaga CL, Lovvorn HN, 3rd, Zent R, Clark PE. Functional KRAS mutations and a potential role for PI3K/AKT activation in Wilms tumors. Mol Oncol. 2017;11(4):405–21.

34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta c(t)) method. Methods 2001;25(4):402–8.

35. Beck JW, Saavedra D, Antell GJ, Tejeiro B. The treatment of pinworm infections in humans (enterobiasis) with pyrvinium chloride and pyrvinium pamoate. Am J Trop Med Hyg. 1959;8(3):349–52.

36. Esumi H, Lu J, Kurashima Y, Hanaoka T. Antitumor activity of pyrvinium pamoate, 6-(dimethylamino)-2-[2-(2,5-dimethyl-1-phenyl-1H-pyrrol-3-yl)ethenyl]-1-methyl-qu inolinium pamoate salt, showing preferential cytotoxicity during glucose starvation. Cancer Sci. 2004;95(8):685–90.

37. Yu DH, Macdonald J, Liu G, Lee AS, Ly M, Davis T, Ke N, Zhou D, Wong-Staal F, Li QX. Pyrvinium targets the unfolded protein response to hypoglycemia and its anti-tumor activity is enhanced by combination therapy. PLoS One 2008;3(12):e3951.

38. Barham W, Frump AL, Sherrill TP, Garcia CB, Saito-Diaz K, VanSaun MN, Fingleton B, Gleaves L, Orton D, Capecchi MR, Blackwell TS, Lee E, Yull F, Eid JE. Targeting the Wnt pathway in synovial sarcoma models. Cancer Discov. 2013;3(11):1286–301.

39. Saraswati S, Alfaro MP, Thorne CA, Atkinson J, Lee E, Young PP. Pyrvinium, a potent small molecule Wnt inhibitor, promotes wound repair and post-MI cardiac remodeling. PLoS One 2010;5(11):e15521.

40. Thorne CA, Lafleur B, Lewis M, Hanson AJ, Jernigan KK, Weaver DC, Huppert KA, Chen TW, Wichaidit C, Cselenyi CS, Tahinci E, Meyers KC, Waskow E, Orton D, Salic A, Lee LA, Robbins DJ, Huppert SS, Lee E. A biochemical screen for identification of small-molecule regulators of the Wnt pathway using Xenopus egg extracts. J Biomol Screen. 2011;16(9):995–1006.

41. Bastakoty D, Saraswati S, Cates J, Lee E, Nanney LB, Young PP. Inhibition of Wnt/beta-catenin pathway promotes regenerative repair of cutaneous and cartilage injury. FASEB J. 2015;29(12):4881–92.

42. Bahrami A, Amerizadeh F, ShahidSales S, Khazaei M, Ghayour-Mobarhan M, Sadeghnia HR, Maftouh M, Hassanian SM, Avan A. Therapeutic potential of targeting Wnt/beta-catenin pathway in treatment of colorectal cancer: Rational and progress. J Cell Biochem. 2017;118(8): 1979–83.

43. Sebio A, Kahn M, Lenz HJ. The potential of targeting Wnt/beta-catenin in colon cancer. Expert Opin Ther Targets 2014;18(6):611–5.

44. Lenz HJ, Kahn M. Safely targeting cancer stem cells via selective catenin coactivator antagonism. Cancer Sci. 2014;105(9):1087–92.

45. Yang L, Wu X, Wang Y, Zhang K, Wu J, Yuan YC, Deng X, Chen L, Kim CC, Lau S, Somlo G, Yen Y. FZD7 has a critical role in cell proliferation in triple negative breast cancer. Oncogene 2011;30(43):4437–46.

46. Ma J, Lu W, Chen D, Xu B, Li Y. Role of Wnt co-receptor LRP6 in triple negative breast cancer cell migration and invasion. J Cell Biochem. 2017;118(9):2968–76.

47. Fu DJ, Zhang L, Song J, Mao RW, Zhao RH, Liu YC, Hou YH, Li JH, Yang JJ, Jin CY, Li P, Zi XL, Liu HM, Zhang SY, Zhang YB. Design and synthesis of formononetin-dithiocarbamate hybrids that inhibit growth and migration of PC-3 cells via MAPK/Wnt signaling pathways. Eur J Med Chem. 2017;127:87–99.

48. Chang TS, Wei KL, Lu CK, Chen YH, Cheng YT, Tung SY, Wu CS, Chiang MK. Inhibition of CCAR1, a coactivator of

beta-catenin, suppresses the proliferation and migration of gastric cancer cells. Int J Mol Sci. 2017;18(2).

49. Chao HM, Huang HX, Chang PH, Tseng KC, Miyajima A, Chern E. Y-box binding protein-1 promotes hepatocellular carcinoma-initiating cell progression and tumorigenesis via Wnt/beta-catenin pathway. Oncotarget 2017;8(2):2604–16.

50. Xia S, Ji R, Zhan W. Long noncoding RNA papillary thyroid carcinoma susceptibility candidate 3 (PTCSC3) inhibits proliferation and invasion of glioma cells by suppressing the Wnt/beta-catenin signaling pathway. BMC Neurol. 2017;17(1):30.

51. He J, Shi J, Zhang K, Xue J, Li J, Yang J, Chen J, Wei J, Ren H, Liu X. Sox2 inhibits Wnt-beta-catenin signaling and metastatic potency of cisplatin-resistant lung adenocarcinoma cells. Mol Med Rep. 2017;15(4):1693–701.

52. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. Science 1998;281(5382):1509–12.

53. Zhang T, Otevrel T, Gao Z, Ehrlich SM, Fields JZ, Boman BM. Evidence that APC regulates survivin expression: A possible mechanism contributing to the stem cell origin of colon cancer. Cancer Res. 2001;61(24):8664–7.

54. Meyer N, Penn LZ. Reflecting on 25 years with MYC. Nat Rev Cancer 2008;8(12):976–90.

55. Perlman EJ, Gadd S, Arold ST, Radhakrishnan A, Gerhard DS, Jennings L, Huff V, Guidry Auvil JM, Davidsen TM, Dome JS, Meerzaman D, Hsu CH, Nguyen C, Anderson J, Ma Y, Mungall AJ, Moore RA, Marra MA, Mullighan CG, Ma J, Wheeler DA, Hampton OA, Gastier-Foster JM, Ross N, Smith MA. MLLT1 YEATS domain mutations in clinically distinctive favourable histology Wilms tumours. Nat Commun 2015;6:10013.

56. Garg H, Suri P, Gupta JC, Talwar GP, Dubey S. Survivin: A unique target for tumor therapy. Cancer Cell Int. 2016; 16:49.

57. Miller MA, Karacay B, Breslow NE, Li S, O'Dorisio MS, Grundy PE, Sandler AD. Prognostic value of quantifying apoptosis factor expression in favorable histology Wilms tumors. J Pediatr Hematol Oncol. 2005;27(1):11–4.

58. Basta-Jovanovic G, Radojevic-Skodric S, Brasanac D, Djuricic S, Milasin J, Bogdanovic L, Opric D, Savin M, Baralic I, Jovanovic M. Prognostic value of survivin expression in Wilms tumor. J BUON 2012;17(1):168–73.

59. Radojevic-Skodric S, Basta-Jovanovic G, Brasanac D, Nikolic N, Bogdanovic L, Milicic B, Milasin J. Survivin gene promoter-31 G/C polymorphism is associated with Wilms tumor susceptibility in Serbian children. J Pediatr Hematol Oncol. 2012;34(8):e310–4.

60. Tao YF, Lu J, Du XJ, Sun LC, Zhao X, Peng L, Cao L, Xiao PF, Pang L, Wu D, Wang N, Feng X, Li YH, Ni J, Wang J, Pan J. Survivin selective inhibitor YM155 induce apoptosis in SK-NEP-1 Wilms tumor cells. BMC Cancer 2012;12:619.