Inhibition of canonical WNT signaling attenuates human leiomyoma cell growth

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Objective: To assess the effect of three WNT/β-catenin pathway inhibitors—inhibitor of β-catenin and TCF4 (ICAT), niclosamide, and XAV939—on the proliferation of primary cultures of human uterine leiomyoma cells.
Design: Prospective study of human leiomyoma cells obtained from myomectomy or hysterectomy.
Setting: University research laboratory.
Patient(s): Women (n = 38) aged 27–53 years undergoing surgery.
Intervention(s): Adenoviral ICAT overexpression or treatment with varying concentrations of niclosamide or XAV939.
Main Outcome Measure(s): Cell proliferation, cell death, WNT/-catenin target gene expression or reporter gene regulation, β-catenin levels, and cellular localization.
Result(s): Inhibitor of β-catenin and TCF4, niclosamide, or XAV939 inhibit WNT/β-catenin pathway activation and exert antiproliferative effects in primary cultures of human leiomyoma cells.
Conclusion(s): Three WNT/-catenin pathway inhibitors specifically block human leiomyoma growth and proliferation, suggesting that the canonical WNT pathway may be a potential therapeutic target for the treatment of uterine leiomyoma. Our findings provide rationale for further preclinical and clinical evaluation of ICAT, niclosamide, and XAV939 as candidate antitumor agents for uterine leiomyoma. (Fertil Steril® 2014;101:1441–9. ©2014 by American Society for Reproductive Medicine.)
Key Words: Leiomyoma, WNT/β-catenin, niclosamide, XAV939, tumor biology

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Uterine leiomyomas, the most common pelvic tumor in women, are benign smooth-muscle tumors originating from the myometrium [1]. Uterine leiomyomas occur in approximately 70% of Caucasian women and more than 80% of African American women by age 50 years and cause excessive uterine bleeding, anemia, recurrent pregnancy loss, preterm labor, pelvic discomfort, and urinary incontinence in approximately 15%–30% of cases [2–4]. Recently it has been reported that ulipristal acetate controlled excessive bleeding due to leiomyoma and reduced the size of the tumor preoperatively [5]. Ulipristal acetate is noninferior to once-monthly leuprolide acetate in controlling uterine bleeding and is significantly less likely to cause hot flashes [6]. However, leiomyomas are still the most common indication for hysterectomy [3, 7–9]. Approximately 200,000 hysterectomies, 30,000 myomectomies, and thousands of selective uterine-artery embolizations and high-intensity focused ultrasound procedures are performed annually in the United States to remove or destroy uterine leiomyoma. The annual economic burden of these tumors is estimated to be between $5.9 billion and $34.4 billion [8, 10]. Thus, there is a need to discover new treatment modalities.

Selective overexpression of constitutively activated β-catenin in uterine mesenchyme during embryonic

development and in adults gives rise to leiomyoma-like tumors in the uterus of all female mice, suggesting that signaling by WNT/β-catenin seems to play a role in somatic stem cell function in the myometrium and uterine leiomyoma tissue (11). WNT signaling through its Frizzled (Fzd) receptor leads to stabilization of cytosolic β-catenin and its translocation to the nucleus, which results in the activation of the transcription factor T-cell factor/lymphoid enhancer factor (TCF/LEF) (12, 13). In the absence of pathway stimulation, a complex consisting of Axin, glycogen synthase kinase 3 (GSK3), and adenomatous polyposis coli promotes β-catenin phosphorylation and subsequent proteolytic degradation.

WNT signaling is important for embryonic development and is also active in stem cells (12, 14, 15). Dysregulation of WNT signaling is a common denominator in a variety of tumors (16–18), and WNT-activating mutations are present in many cancers, including colon cancer, hepatocellular carcinoma, melanoma, non–small-cell lung cancer, and ovarian endometrioid cancer (16). WNT ligands or inhibitors have been shown to affect the growth and survival of colon cancer cells (19–21), suggesting that targeting WNT pathway receptors may be a more feasible therapeutic approach than interfering with downstream protein–protein interactions. Indeed, blocking canonical WNT activity in colorectal and other WNT-deregulated cancers causes cell-cycle arrest in the G_1 phase, leading to tumor cell growth inhibition (22, 23).

It is well established that WNT/β-catenin signaling can promote cell proliferation, particularly in epithelial-derived cancers and stem cell populations (24–26), but whether β-catenin signaling drove smooth-muscle cell proliferation through activation of similar gene targets as epithelial cells was unclear. Recently it was reported that lung fibroblasts proliferated under the control of β-catenin signaling (27, 28). In recent years, several classes of small molecules have been shown to act as WNT inhibitors, which exert their inhibitory effects at various levels of the WNT signaling pathway (29–35). Three small molecules—inhibitor of β-catenin and TCF4 (ICAT), niclosamide, and XAV939—have been shown to strongly and specifically reduce levels of active β-catenin in vitro by inhibiting β-catenin stabilization and downstream β-catenin signaling (TCF/LEF reporter activity). In this study we hypothesized that these molecules also inhibit WNT/β-catenin signaling to reduce the growth and proliferation of human leiomyoma cells. We tested the effects of these molecules on primary cultures of leiomyoma cells from women undergoing hysterectomy or myomectomy for leiomyoma, with the goal of establishing the antitumor properties of these molecules as potential candidates for further development.

MATERIALS AND METHODS
Preparation of Human Leiomyoma Cells

Leiomyoma samples were obtained from surgeries from 38 women (age range, 27–53 years) undergoing hysterectomy or myomectomy. We used leiomyomas derived from patients without receiving hormonal treatment. Deidentified patient information was collected, including age, weight, height, race, day of last menstrual cycle, parity, and use of oral contraceptives. Written informed consent was obtained from each patient, and the use of human tissue was approved by the Institutional Review Board for Human Research at Northwestern University, Chicago, Illinois. None of the women had a previous history of uterine cancer, and all samples were confirmed by histopathologic examination to be free of malignancy.

Cell Culture and Treatment

We isolated leiomyoma cells using a technique that was previously described (35, 36). We used α-smooth muscle actin protein expression and *MED12* gene mutations as a marker for leiomyoma cells. Leiomyoma cells were cultured in 96-well flat-bottomed plates in Dulbecco's modified eagle medium (DMEM)/F12 1:1 (Life Technologies) containing 10% fetal bovine serum and grown in a humidified atmosphere with 5% CO_2 at 37°C. Freshly cultured leiomyoma cells were treated with 30 mmol/L of NaCl (Sigma) for the baseline β-catenin condition or 30 mmol/L of LiCl (Sigma) to inhibit GSK-3β activity, thereby activating β-catenin (37). For cell proliferation assays, leiomyoma cells were incubated for 3 days with various concentrations of niclosamide (0.4, 2, or 10 μmol/L; Sigma) or XAV939 (0.1, 1, 10, or 20 μmol/L; R&D Systems). Reagents were diluted with dimethyl sulfoxide (DMSO; Sigma). Control cells were treated with DMSO.

ICAT Adenoviral Transduction

Leiomyoma cells were cultured in 96-well flat-bottom plates and infected with adenovirus encoding green fluorescent protein (Ad-GFP) and Ad-ICAT for 72 hours. The myc-ICAT complementary DNA was provided by Tetsu Akiyama (University of Tokyo) and re-engineered by Vector Biolabs to encode a bicistronic messenger RNA that translates myc-ICAT and GFP proteins [Ad-ICAT (Myc)-Internal Ribosome Entry Site-GFP], and Ad-GFP viruses were used as a control for ICAT treatment and supplied by Tong Chuan He (University of Chicago). Infection efficiency of >90% was confirmed by visualizing GFP expression in living cells. Leiomyoma cells without adenoviral infection were used as controls.

Cell Proliferation Assay

After viral transduction, cells were incubated for 3 days. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) was used according to the manufacturer's instructions. Proliferation was measured as the optical density (OD) at 490 nm on a Synergy-HT plate reader (Bio-Tek Instruments).

Lactate Dehydrogenase Cell Death Assay

Leiomyoma cell death was assayed as previously described (38). Lactate dehydrogenase (LDH) activity was measured using a Cytotoxicity Detection Kit (Roche). The OD_{490} was read

using a Synergy-HT plate reader and software (Bio-Tek Instruments).

Quantitative Real-time Reverse Transcription–polymerase Chain Reaction

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Complementary DNA was synthesized from 1 μg of RNA using qScript cDNA SuperMix (Quanta Biosciences). Quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) of the WNT target genes *AXIN2* (NM_004655) and Naked 1 (*NKD1*) (NM_033119) was performed using the ABI 7900HT (Life Technologies) and Power SYBR Green PCR Master Mix (Life Technologies) for 40 cycles, with each cycle consisting of a 15-second denaturation at 95.0°C, followed by 1 minute of annealing at 60.0°C. The sizes of amplicons were 102 bp and 99 bp for *AXIN2* and *NKD1*, respectively. The specificity of each PCR product was analyzed using the built-in melting curve analysis tool for each DNA product identified. For any sample, the expression level, normalized to the housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (NM_002046), was determined with the comparative threshold cycle (Ct) method.

Transfections and β-Catenin/TCF Reporter Assay

Leiomyoma cells were transfected with a TCF optimal luciferase promoter plasmid (TOPflash; containing four consensus TCF-binding sites upstream of a minimal c-fos promoter) or FOPflash (containing four mutant TCF-binding sites; kindly provided by H. Clevers, Utrecht, the Netherlands), using Lipofectamine 2000 (Invitrogen). A thymidine kinase–Renilla plasmid (50 ng) was cotransfected to normalize luciferase values to transfection efficiency. Cells were treated with niclosamide or XAV939, with or without LiCl, 24 hours after transfection. Cells were solubilized 2 days after transfection using the Dual-Luciferase Assay Kit (Promega), and luciferase activity was quantified with a Synergy-HT plate reader (Bio-Tek Instruments). Firefly luciferase readings were first normalized to the reading for the corresponding Renilla luciferase reading to normalize for transfection efficiency. The adjusted TOPflash reading was then normalized to the corresponding adjusted FOPflash reading to account for background reading of the TOPflash construct.

Immunofluorescence Staining

Indirect immunofluorescence staining was performed as described previously (35, 36, 39). Background fluorescence was determined by applying the secondary conjugated antibody alone and by replacement of the primary antibody with nonimmune serum. Slides were successively stained with β-catenin antibody (clone 14; 1:100; BD Biosciences), followed by incubation with secondary antibodies labeled with Alexa Fluor 488 Dye (Molecular Probes Life Technologies). Images were collected using a confocal laser scanning system (LSM 510; Karl Zeiss).

Cadherin-free β-catenin–binding Assays

To evaluate activation of canonical WNT signaling, intracellular levels of cadherin-free β-catenin were determined by pull-down assays using glutathione S-transferase (GST)/ICAT, as previously described (40). At the end of the treatment period, cells were harvested, solubilized in a nonionic detergent buffer (1% Nonidet P40, 50 mM Tris [pH 7.5], 150 mM NaCl, and 2 mM ethylenediaminetetraacetic acid including protease inhibitors) and centrifuged at 14,000 × *g*. Cellular β-catenin was affinity-precipitated using GST-ICAT immobilized to glutathione-coupled Sepharose (Sigma), washed with buffer A (10 mM Tris [pH 8.0], 140 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.1% Nonidet P40, and 10 μg/mL leupeptin and aprotinin), and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblot analysis using β-catenin, ICAT (polyclonal; Santa Cruz Biotechnology) and β-actin antibody (clone; AC-15, Sigma).

Statistical Analysis

Each experiment was repeated using cells from at least three subjects, followed by statistical analysis. All of the data were expressed as the mean ± SD. Probability (*P*) values were calculated using the Student *t* test. Values of $P < .05$ were considered statistically significant.

RESULTS

Adenoviral Overexpression of ICAT Inhibits Human Leiomyoma Cell Proliferation

Because leiomyoma proliferation is thought to be a critical step in leiomyoma tumorigenesis, we addressed the contribution of WNT/β-catenin signaling to these phenotypes using cultures of human leiomyoma cells and adenoviruses (Ad-ICAT), which can inhibit WNT/β-catenin signaling. Human leiomyoma cells were transduced with Ad-GFP or Ad-ICAT for 72 hours. After viral transduction, cells were incubated for 3 days. Cell proliferation was measured by MTS assay (n = 3). Leiomyoma cells transduced with Ad-ICAT exhibited significantly lower proliferation compared with Ad-GFP transduced cells (Supplemental Fig. 1A, available online). The observed reduction in proliferation with ICAT overexpression was not due to diffuse cellular toxicity, becaise Ad-GFP or Ad-ICAT transduced leiomyoma cells showed no difference in LDH levels compared with the control group (No Tx; Supplemental Fig. 1B).

Overexpression of ICAT Inhibits Expression of WNT/β-catenin Target Genes in Human Leiomyoma Cells

Leiomyoma cells (n = 4) were treated with LiCl for 24 hours to induce β-catenin and transcription of WNT pathway target genes *AXIN2* and *NKD1* (Supplemental Fig. 1C and D). Subsequent adenoviral expression of ICAT significantly inhibited LiCl-stimulated *AXIN2* and *NKD1* gene expression, measured by real-time PCR. Ad-ICAT transduced cells without LiCl treatment had messenger RNA expression levels of *AXIN2* and *NKD1* similar to those in Ad-GFP transduced cells.

Niclosamide Treatment Inhibits Human Leiomyoma Cell Proliferation

Niclosamide has been shown to inhibit WNT pathway activation in colorectal cancer cells (41, 42). Human leiomyoma cells (n = 4) were treated with DMSO or increasing concentrations of niclosamide for 3 days before analysis by MTS assay. Niclosamide inhibited proliferation of leiomyoma cells in a dose-dependent manner (Fig. 1A and B). The observed reduction in proliferation after niclosamide treatment was not related to decreased cell survival: LDH activity levels were similar for leiomyoma cells in all treatment groups (Fig. 1C).

Niclosamide Inhibits WNTβ-catenin Pathway Activation in Human Leiomyoma Cells

Activation of the WNT/β-catenin pathway results in stimulation of the TCF/LEF transcription factor, which binds to TCF elements in the promoters of target genes (12, 13). To analyze the effects of niclosamide on WNT/β-catenin signaling in human leiomyoma cells, we transfected cells (n = 6) with luciferase reporter constructs consisting of four copies of the TCF element (TOPflash), then treated the cells with increasing concentrations of niclosamide in the presence of LiCl. As expected, LiCl treatment (activates β-catenin) up-regulated TOPflash reporter activity. This effect was blunted by treatment with increasing concentrations of niclosamide (Fig. 2A). Niclosamide treatment also down-regulated LiCl-induced AXIN2 and NKD1 expression in human leiomyoma cells (n = 4) (Fig. 2B).

Activation of the WNT signaling pathway results in the accumulation of β-catenin in the nucleus, followed by heterodimer formation with TCF and activation of TCF-dependent transcription (12, 13). We examined the effects of niclosamide on subcellular localization of β-catenin in human leiomyoma cells (n = 5) (Fig. 2C). Treatment with LiCl induced nuclear accumulation of β-catenin, whereas treatment with niclosamide blocked both baseline and LiCl-induced β-catenin nuclear translocation. We evaluated the amount of β-catenin in human leiomyoma cells (n = 3) with GST-ICAT pull-down assays. As expected, β-catenin levels were significantly lower in niclosamide-treated leiomyoma cells compared with vehicle-treated cells (Fig. 2D).

Tankyrase Inhibitor (XAV939) Disrupts Leiomyoma Cell Proliferation

Tankyrases 1 and 2 are proteins with poly (adenosine diphosphate–ribose) polymerase activity. Tankyrases have been shown be involved in the WNT signaling pathway. Tankyrases bind directly to AXIN, a member of the "destruction complex" involved in β-catenin degradation. Inhibition of tankyrase stabilizes AXIN, increases the activity of the destruction complex, and promotes degradation of β-catenin. XAV939 is a tankyrase inhibitor, thereby inhibiting WNT/β-catenin signaling. Human leiomyoma cells (n = 6) were treated with DMSO or increasing concentrations of XAV939 for 3 days before analysis by MTS assay. XAV939 inhibited


FIGURE 1
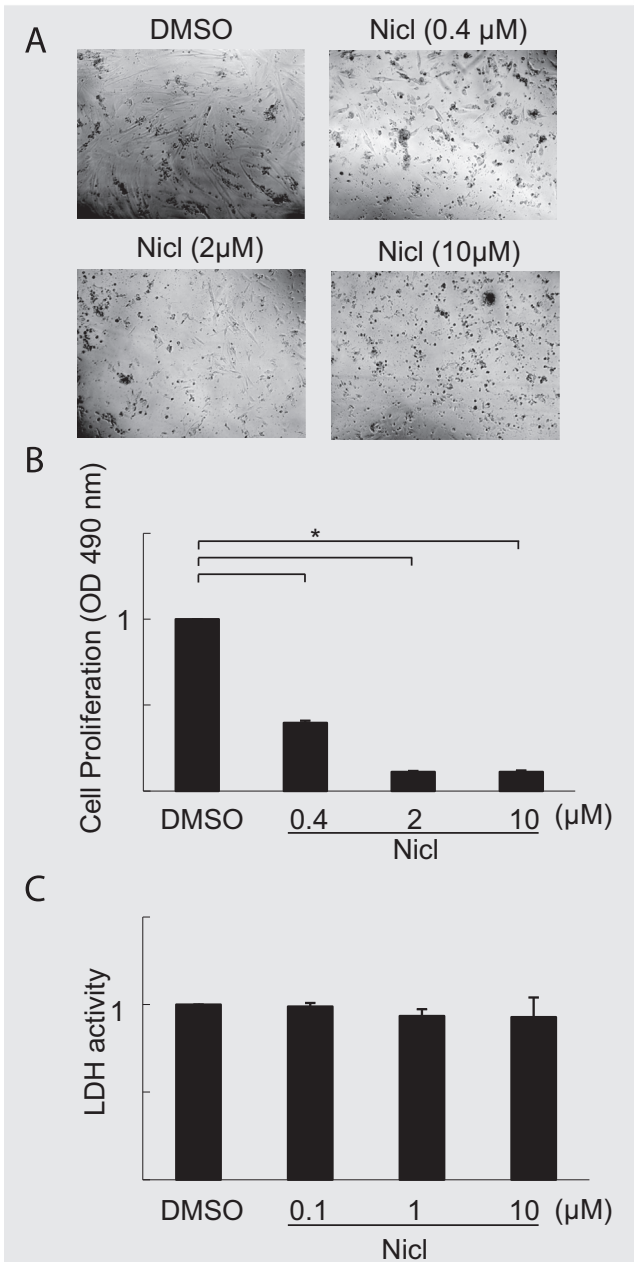
Niclosamide effect on proliferation of human leiomyoma cells. (A) Primary cultures of human leiomyoma cells after treatment for 3 days with DMSO or 0.4, 2, or 10 μM niclosamide (Nicl). (B) Leiomyoma cell proliferation at different doses of niclosamide. Niclosamide inhibits leiomyoma cell proliferation in a dose-dependent manner. Cell proliferation was measured by MTS assay and reported as the OD at 490 nm. Results are expressed as mean OD 490 nm ± SD from four independent experiments. (C) Cell viability was assessed in terms of LDH activity in the culture medium. Niclosamide does not promote human leiomyoma cell death, with no difference in LDH activity across treatment groups. Results are expressed as mean levels of LDH activity ± SD from three independent experiments. *P<.05.

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the proliferation of leiomyoma cells in a dose-dependent manner (Fig. 3A and B). The reduced proliferation observed with XAV939 was not due to an effect on diffuse cell toxicity,

FIGURE 2
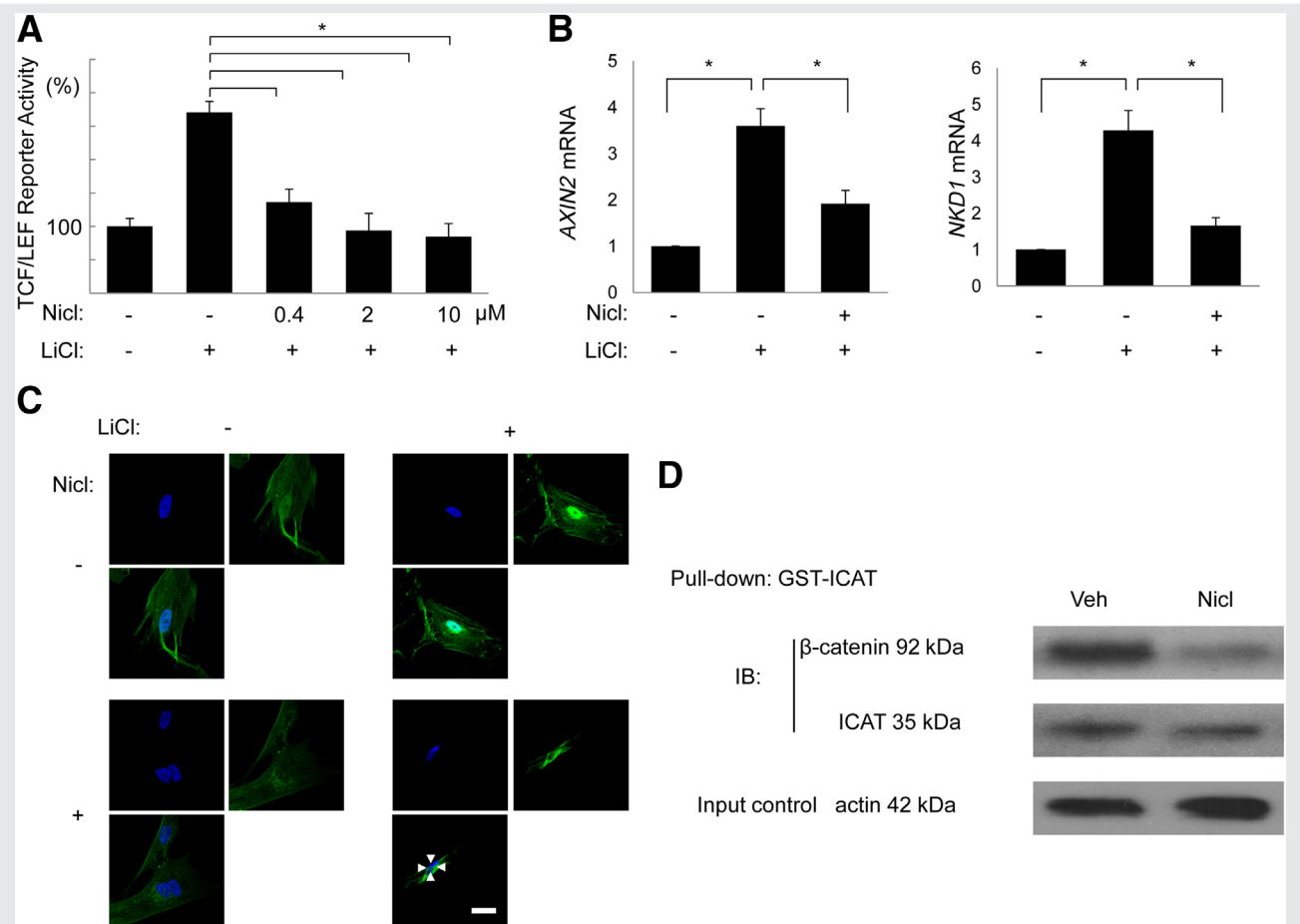
Niclosamide effect on WNT/β-catenin signaling in human leiomyoma cells. (A) Niclosamide effect on LiCl-induced WNT/β-catenin/TCF-responsive reporter activation. Cells were transiently cotransfected with cytomegalovirus-Renilla luciferase construct (pRL–CMV) and either the TOPflash or FOPflash Firefly luciferase reporter plasmid before treatment with LiCl and/or niclosamide for 24 hours. Each reporter plasmid/condition was transfected into cells plated in triplicate wells, and data are reported as the mean TOPflash activity normalized for transfection efficiency (pRL) and construct background (FOPflash) ± SD for six independent experiments. (B) Niclosamide treatment and LiCl-induced WNT/β-catenin target gene expression in human leiomyoma cells. Cells were treated with NaCl or LiCl to activate β-catenin and induce downstream expression of WNT/β-catenin target genes *AXIN2* and *NKD1*. Total RNA was extracted for quantitative real-time PCR of *AXIN2* and *NKD1* transcripts. Results are expressed as the mean expression ± SD of *AXIN2* and *NKD1* relative to *GAPDH* from four independent experiments. (C) Niclosamide effect on nuclear localization of β-catenin in human leiomyoma cells. Indirect immunofluorescence was performed on leiomyoma cells in the presence or absence of LiCl and niclosamide using an anti–β-catenin antibody (*green*), followed by Hoechst nuclear counterstaining (*blue*). Representative images from five independent experiments; scale bar = 20 μm. *Arrowheads* indicate nucleus. (D) Niclosamide effect on β-catenin levels in human leiomyoma cells. Whole-cell lysates from leiomyoma cells treated with DMSO or niclosamide were affinity precipitated with GST-ICAT to pull down cytosolic β-catenin. Active β-catenin bound to ICAT was probed with an anti–β-catenin antibody. Representative immunoblots from three independent samples are shown. Actin loading controls were taken from inputs. *$P<.05$.

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because LDH activity levels were the same across treatment groups (Fig. 3C).

XAV939 Inhibits WNT/β-catenin Pathway Activation in Human Leiomyoma Cells

To analyze the effect of XAV939 on WNT/β-catenin signaling in human leiomyoma cells (n = 6), we transfected cells with the TCF reporter constructs (TOPflash or FOPflash) and treated the cells with increasing concentrations of XAV939 in the presence of LiCl. XAV939 inhibited LiCl-induced TCF reporter activation in a dose-dependent manner (Fig. 4A). XAV939 also down-regulated LiCl-induced *AXIN2* and *NKD1* expression

in human leiomyoma cells, measured by real-time quantitative PCR (Fig. 4B). XAV939 significantly inhibited LiCl-stimulated nuclear translocation of β-catenin in human leiomyoma cells (Fig. 4C). The GST-ICAT pull-down assays revealed a remarkable decrease in active β-catenin upon treatment of human leiomyoma cells with XAV939 (Fig. 4D).

DISCUSSION

Because many β-catenin target genes are involved in cell proliferation, we hypothesized that enhanced WNT/β-catenin signaling in leiomyoma cells contributes to leiomyoma tumor growth in humans. Notably, human leiomyoma cell primary

FIGURE 3

XAV939 (XAV) and proliferation of human leiomyoma cells. (A) Cultured primary leiomyoma cells after treatment for 3 days with DMSO or 0.1, 1, 10, or 20 μM XAV939. (B) XAV939 inhibits leiomyoma cell proliferation in a dose-dependent manner, measured by MTS assay. Results are expressed as mean OD 490 nm $\pm$ SD from six independent experiments. (C) XAV939 does not affect human leiomyoma cell survival. LDH activity in the culture medium did not differ between treatment groups. Results are expressed as mean levels of LDH activity $\pm$ SD from six independent experiments. *P<.05.

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cultures contain a substantial level of GST-ICAT–precipitable β-catenin at baseline (Figs. 2D and 4D). Activation of β-catenin signaling has been spontaneously observed in a number of primary cell cultures (38, 43). We tested whether the inhibition of WNT/β-catenin signaling alters human leiomyoma cell proliferation. Results showed that ICAT, niclosamide, and XAV939 specifically inhibited canonical WNT/β-catenin signaling and decreased WNT-dependent transcription in primary cultures of human leiomyoma cells, which resulted in reduced cell growth and proliferation in vitro.

Our findings add to the existing body of knowledge on the critical role that the WNT/β-catenin signaling pathway plays in not only development and adult tissue homeostasis but also tumor development and fibrogenesis (11, 27, 44, 45). Selective deletion of β-catenin in uterine mesenchyme during embryonic development significantly reduces uterine size and replaces it with adipocytes, thus completely disrupting normal myometrial smooth-muscle differentiation and regeneration (35, 36, 46, 47). Conversely, selective overexpression of constitutively activated β-catenin in uterine mesenchyme during embryonic development and in adults gives rise to leiomyoma-like tumors in the uterus of all female mice (11). This suggests that β-catenin plays a key role in stem cell renewal and differentiation to the smooth-muscle phenotype observed in myometrial and leiomyoma tissues, and that the WNT/β-catenin pathway may be promising target for new therapeutics (48).

Uterine leiomyomas are monoclonal tumors, with growth of the neoplasm occurring via clonal expansion from a single cell; this raises the possibility that interventions targeted at the mechanisms that underlie cell expansion may be particularly effective in the treatment of uterine leiomyomas (49). Components of the WNT pathway are prime drug development targets, particularly the accessible plasma membrane receptors such as Frizzled. Vuga et al. (50) found that WNT5a can increase fibroblast proliferation through a "noncanonical" or β-catenin/TCF-independent signaling mechanism, indicating that both canonical and noncanonical WNTs may contribute to tumorigenesis. Given that WNT/β-catenin target genes are cell-type and cell-context dependent, identifying the WNT/β-catenin–regulated target genes in human leiomyoma requires similar unbiased approaches.

Most leiomyomas contain specific genetic mutations, such as those found in MED12, suggesting that transformation of normal myocytes into abnormal myocytes is required at some point during the genesis of a leiomyoma (51). MED12 binds directly to β-catenin and regulates canonical WNT signaling (52). Interestingly, expression of WNT4, an activator of β-catenin, is markedly elevated in leiomyoma with MED12 mutations vs. those without mutations (44). These observations point to a mechanism involving MED12 mutations and WNT/β-catenin activation that supports cell proliferation in uterine leiomyoma tissue (52, 53).

Interestingly, the size and number of β-catenin–driven leiomyoma-like tumors increases with parity in mice, suggesting that exposure to steroid hormones may interact with activated β-catenin to accelerate tumorigenesis. Moreover, activated β-catenin induces expression of transforming growth factor-β3, which induces proliferation and extracellular matrix formation in human leiomyoma tissue (11, 54). It is known that β-catenin signaling antagonizes adipogenesis by inhibiting the adipogenic transcription factors CCAAT/enhancer binding protein-α and peroxisome proliferator-activated receptor γ (55). Two percent of all leiomyomas contain adipocytes that have undergone metaplasia.

FIGURE 4
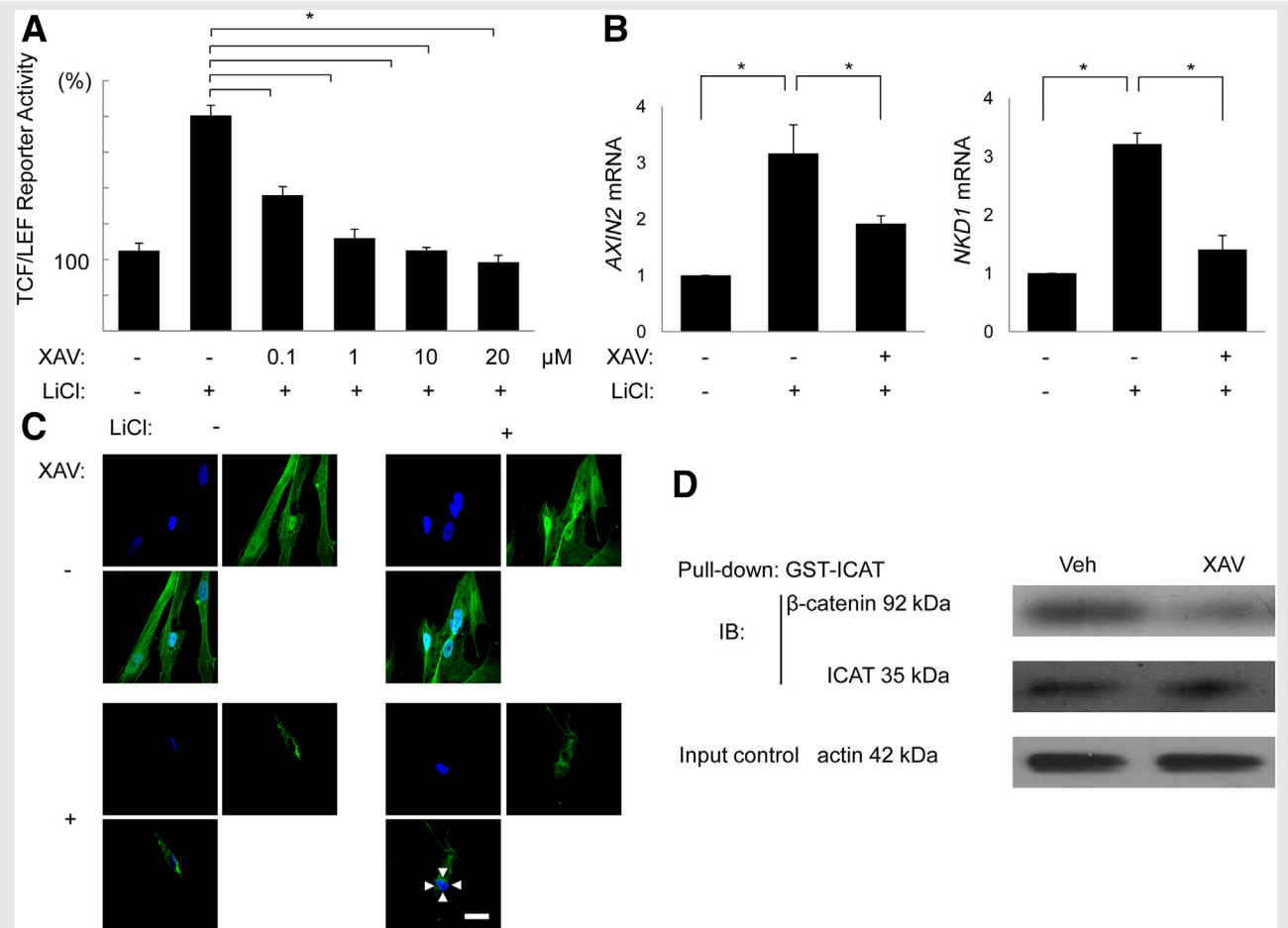
XAV939 and WNT/β-catenin signaling in human leiomyoma cells. (A) XAV939 effect on LiCl-induced WNT/β-catenin/TCF-responsive reporter activation. Cells were transiently cotransfected with pRL-CMV and either the TOPflash or FOPflash Firefly luciferase reporter plasmid before treatment with LiCl and/or XAV939 for 24 hours. Each reporter plasmid/condition was transfected into cells plated in triplicate wells, and data are reported as the mean TOPflash activity normalized for transfection efficiency (pRL) and construct background (FOPflash) $\pm$ SD for six independent experiments. (B) XAV939 and inhibits LiCl-induced WNT/β-catenin target gene expression in human leiomyoma cells. Cells were treated with NaCl or LiCl to activate β-catenin and induce downstream expression of WNT/β-catenin target genes *AXIN2* and *NKD1*. Total RNA was extracted to determine expression of *AXIN2* and *NKD1* by quantitative real-time PCR. Results are expressed as the mean expression $\pm$ SD of *AXIN2* and *NKD1* relative to *GAPDH* from four independent experiments. (C) XAV939 treatment effect on nuclear localization of β-catenin in human leiomyoma cells. Indirect immunofluorescence was performed on leiomyoma cells after treatment with NaCl or LiCl and DMSO or XAV939, using an anti–β-catenin antibody (*green*) followed by Hoechst nuclear counterstaining (*blue*). Merged images of β-catenin (*green*) and Hoechst (*blue*) staining are also shown. Representative images from five independent experiments; scale bar = 20 μm. *Arrowheads* indicate nucleus. (D) XAV939 treatment and β-catenin levels in human leiomyoma cells. Whole-cell lysates from leiomyoma cells treated with DMSO or XAV939 were affinity precipitated with GST-ICAT to isolate cytosolic β-catenin. Active β-catenin bound to GST-ICAT was probed with an anti–β-catenin antibody. Representative immunoblots from three independent samples are shown. Actin loading controls were taken from inputs. *$P<.05$.

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This suggests that aberrant β-catenin signaling affects the tumor initiation and tumor maintenance in uterine leiomyoma.

The mechanism of action of niclosamide is thought to be through the internalization of Fzd1 and down-regulation of WNT pathway intermediaries (41). In its common usage as an antihelminthic, it is believed to uncouple oxidative phosphorylation (56, 57). It has recently been found to be effective at low micromolar concentrations in preventing the synthesis of corona virus proteins in a tissue culture model of severe acute respiratory syndrome (58). It is known that the function of β-catenin is controlled by

ubiquitination and ultimately degradation by the proteasome; a possible mechanism of niclosamide function might be its effect on either the ubiquitination or a proteolytic pathway to cause degradation of β-catenin (59). Recent studies found that XAV939 inhibits the Poly (ADP-ribose) polymerase (PARP) domain of tankyrase 1 and 2 and destabilizes AXIN1 and 2 by Poly-ADP-ribosylation (PARsylation) (60). In the present study we showed by TOPflash assay that niclosamide and XAV939 can inhibit WNT pathway activation in leiomyoma cells. We attempted to rule out nonspecific toxic effects of niclosamide or

XAV939 on cells using the LDH assay. We noticed morphologic changes in these cells after treatments. Thus, further studies will be needed to assess nonspecific toxic effects of these compounds on both diseased and healthy tissues.

Niclosamide and XAV939 were administered orally in the referenced reports (42, 61). It can be speculated that the systemic treatment works because the WNT canonical signaling is highly active in the diseased tissue, therefore it is more susceptible to the effects of the drugs compared with healthy tissue, allowing for a lower dose to be administered. However, reducing undesirable side effects should always be a goal when developing novel therapies. Consequently, to avoid any off-target effects, it might be attractive to develop a vaginal delivery system, which can deliver therapeutics locally and avoid systemic complications.

In summary, we showed that the down-regulation of WNT/β-catenin signaling, via ICAT, niclosamide, or XAV939, has antitumor effects on primary cultures of human leiomyoma cells in vitro. Our study demonstrates that sustained activation of WNT/β-catenin signaling in leiomyoma cells can enhance activities associated with leiomyoma formation, such as proliferation. However, given that WNT/β-catenin signaling controls cell-fate decisions throughout development, often controlling the balance between progenitor cells and their descendants, we speculate that cells serving as progenitors to leiomyoma may be a key target of WNT/β-catenin signaling in leiomyoma tumorigenesis (62).

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SUPPLEMENTAL FIGURE 1
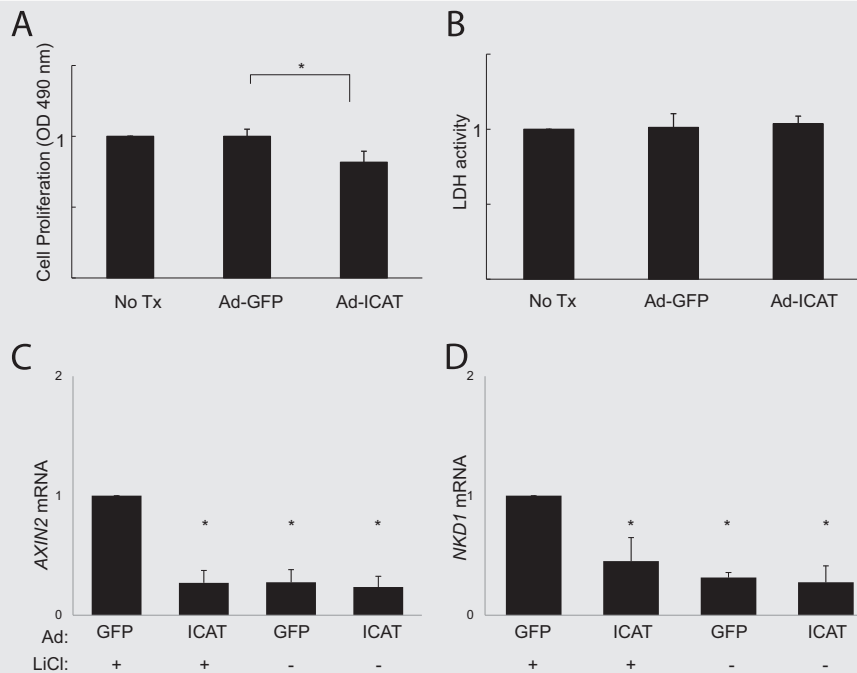
Effect of ICAT on proliferation and WNT/β-catenin target gene expression in human leiomyoma cells. (A) Overexpression of ICAT effect on leiomyoma cell proliferation. Results are expressed as mean OD 490 nm $\pm$ SD from three independent experiments. (B) Cell viability was assessed in terms of LDH activity. Inhibition of WNT/β-catenin signaling with overexpression of ICAT does not promote human leiomyoma cell death, with no difference in LDH activity compared with control transduced (Ad-GFP) or nontransduced (No Tx) cells. Results are expressed as mean levels of LDH activity $\pm$ SD from six independent experiments. (C, D) Overexpression of ICAT in human leiomyoma cells blocks LiCl-induced WNT/β-catenin target gene expression. Cells were treated with NaCl or LiCl to activate β-catenin and induce downstream expression of WNT/β-catenin target genes AXIN2 and NKD1. Total RNA was extracted for quantitative real-time PCR of AXIN2 and NKD1 transcripts. Results are expressed as the mean expression $\pm$ SD of AXIN2 and NKD1 relative to GAPDH from four independent experiments. *P<.05. No Tx = nontransduced cells; Ad-GFP = control transduced cells; Ad-ICAT = Ad-ICAT transduced cells.

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