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Activation of AKT by WNT4 as a regulator of uterine leiomyoma stem cell function

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Abstract

Objective—To investigate the functional interaction between the WNT/β-catenin and AKT pathways in leiomyoma stem cells (LSC).

Design—Cells from human leiomyoma tissues were sorted by FACS into three populations: LSC, intermediate cells (LIC), and differentiated cells (LDC). The function of the WNT/β-catenin and AKT signaling pathways in leiomyoma cells was evaluated using real-time qPCR and immunoblot analyses.

Setting—Research laboratory.

Patients—Premenopausal women (n = 36, age 28 to 49 years) undergoing hysterectomy or myomectomy for leiomyoma.

Interventions—None.

Main Outcome Measure—Gene expression, protein phosphorylation, and cell proliferation.

Results—WNT/β-catenin signaling pathway components were differentially expressed in each leiomyoma cell population. WNT4 was distinctly overexpressed in LIC, while its receptor FZD6 was primarily expressed in LSC. WNT4 stimulated AKT phosphorylation, activated β-catenin, and increased primary leiomyoma cell proliferation. These stimulatory effects were abolished by co-treatment with the AKT inhibitor, MK-2206. WNT4 upregulated the expression of pro-proliferative genes, c-Myc and cyclin D1 specifically in LSC; this was also abrogated by AKT inhibition.

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Conclusions—Our data suggest that WNT4 regulates LSC proliferation via AKT-dependent β-catenin activation. These findings represent a key step towards a better understanding of leiomyoma stem cell regulation and may reveal novel therapeutic targets.

Keywords

Uterine leiomyoma; AKT; WNT/β-catenin; Stem cell function

Introduction

Uterine leiomyomas (LM) are non-malignant smooth muscle tumors originating from the myometrium of the uterus (1). LM is the most common tumor in women, affecting approximately 80% of women by the age of 50 years (2, 3). LM can cause significant morbidity, including excessive uterine bleeding, recurrent pregnancy loss, and pelvic pain; these symptoms may also mimic or mask malignant tumors (3). Due to the large population affected by LM, more than 200,000 surgeries are performed in the US annually at an estimated cost of $5.9–34.4 billion (4, 5). Despite their prevalence and impact on women's health, no new medical treatments have been approved in the US for LM since the 1990s (6).

Previous studies have shown that a small stem cell-like population is responsible for the initiation of LM (7–10). LM stem cells (LSC) were first identified as a side population based on Hoechst-3342 dye extrusion, a universal feature of somatic stem cells (8, 9, 11). To avoid the toxicity of Hoechst staining, the LSC isolation protocol was refined using flow cytometry to sort LM cells based on the expression of the cell surface markers, CD34 and CD49b (7). Using this approach, three functionally and molecularly distinct LM populations were identified, with a possible hierarchical differentiation order: LSC, LM intermediate cells (LIC), and terminally differentiated LM cells (LDC) (12). While the majority of cells in a LM tumor mass are LDC, LSC are indispensable for proliferation, regeneration, and robust tumor growth (12, 13). LIC express high levels of progesterone receptor (PR), cytokines (RANKL), growth factors (IGF2), and extracellular matrix (ECM) proteins, and play a critical role in transducing progesterone-mediated paracrine mitotic signals to PR-deficient LSC to stimulate their self-renewal and proliferation (12, 13).

β-catenin regulates various cellular processes. In its basal state, β-catenin mainly interacts with E-cadherin and is involved in the regulation of cell-cell adhesion (14). In the presence of canonical WNT signals, activated β-catenin translocates to the nucleus and upregulates the expression of target genes including c-Myc, WISP1, and cyclin D1, to promote cell proliferation (14, 15). We and others have demonstrated the role of WNT/β-catenin pathway in LM tumorigenesis. Constitutive activation of β-catenin in the uterine stroma and muscle cells leads to LM tumor development (15). Somatic mutation of mediator complex subunit 12 (MED12), occuring in 70% of LM, also dysregulates the WNT/β-catenin pathway in LM (16, 17). Using immortalized human uterine fibroid and myometrial cell lines, studies have shown that MED12 regulates the protein levels of WNT4 and β-catenin as well as cell proliferation (18, 19). WNT ligands, secreted by adjacent mature LM or myometrial cells in response to steroid hormones, estrogen and progesterone, acts in a paracrine fashion to induce nuclear translocation of β-catenin and proliferation of LM stem cells, leading to

tumor regeneration in the xenograft mouse model (20). Most recently, Ali et al reported that estradiol and histone deacetylase regulate LM cell function through regulating β-catenin nuclear translocation (21).

The AKT pathway also plays various roles in tumor development, including LM (22, 23). The phosphatidylinositol 3-kinase (PI3K)/AKT pathway is activated by IGF2 to regulate LSC proliferation (12). Various factors, including oxidative stress and progestins, mediate AKT activation which regulates LM cell survival and senescence (24–28). The AKT inhibitor, MK-2206 can also effectively reduce the proliferation of LM cells (29, 30). Studies have reported that the crosstalk between AKT and β-catenin influences the regulation of cell proliferation and disease development. Phosphorylation of β-catenin at Ser 552 by AKT stabilizes β-catenin, increases its transcriptional activity, and promotes tumor growth (31, 32). On the other hand, WNT1 induces AKT phosphorylation and activation through Dishevelled activation (33–35). While evidence suggests roles for the WNT/β-catenin and AKT pathways in LM function (20, 30), in what way these pathways interact in LM pathogenesis remains unclear.

In this study, we explored the interaction between these pathways in primary LM cells and LSC sorted from human LM tissue. We tested the hypothesis that WNT4 regulates LSC proliferation via activation of an AKT-dependent β-catenin signaling pathway.

Materials and Methods

Tissue collection

Northwestern University's Institutional Review Board approved the use of human tissue. LM tissues were obtained from premenopausal women undergoing either myomectomy or hysterectomy (age 38 ± 9 years, range 28–49 years). We obtained informed consent from all participants. Patients receiving hormone treatment within six months prior to surgery were excluded. Tissues were dissociated and the cell populations isolated as previously described (7).

Primary LM cell culture

Primary LM cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic in a humidified atmosphere with 5% CO_2 at 37°C. All primary cells used in this study were within two passages.

Cell treatments

Cells were treated with WNT4 (R&D Systems, 6076-WN-005/CF), SC79 (Millipore, 123871–25MG), or MK-2206 (Selleck Chemicals, S1078) as described. The treatment was performed under starvation conditions, that is, the cells were incubated with phenol-red free DMEM/F12 media containing 0.2% charcoal stripped FBS (cFBS) and 1% antibiotic/antimycotic for 24 hours before treatment. To examine phosphorylation signal changes induced by WNT4, cells were treated with vehicle (0.1% BSA in PBS) or WNT4 (200 ng/ml) for 30 min, if not indicated specifically. To examine proliferation-related changes

induced by WNT4, cells were treated with vehicle (0.1% BSA in PBS) or WNT4 (200ng/ml) for 1h for mRNA evaluation, 24h for protein evaluation, or 96h for Cell Counting Kit-8 (CCK8) proliferation assay described below. To evaluate the crosstalk between WNT4 and AKT pathway, MK-2206 (1 μM) or vehicle (DMSO) were added to the cells 24h prior to WNT4 treatment. To evaluate the crosstalk between AKT and β-catenin pathways, cells were treated with AKT activator, SC79 (10 μM) or vehicle (DMSO) for 15 min, 30min, 60min, 120min, and 360 min.

LM spheroid 3D culture

Freshly FACS-sorted LM populations (LSC, LIC, and LDC) were cultured in mesenchymal stem cell growth medium (Lonza, PT-3238) containing provided supplements and 1% antibiotic/antimycotic in low-attachment 96-well plates under a humidified atmosphere with 5% CO_2 at 37°C normoxia condition. This culture condition maintains the stem cell characteristics of LSC without affecting the viability of all LM cells (30, 36). Cells were recovered in basal mesenchymal stem cell growth medium for three days before treatment.

Antibodies and primers

All antibodies and primers used in this manuscript are listed in Supplementary Tables 1 and 2.

Antibody-based cell sorting

$CD34^+/CD49b^+$ (LSC), $CD34^+/CD49b^-$ (LIC), and $CD34^-/CD49b^-$ (LDC) were FACS-sorted from dissociated LM cells, as previously described (7, 13). Briefly, dissociated leiomyoma cells were resuspended at a concentration of 2 X 10^6 cells/mL in Hanks balanced salt solution containing 2% FBS (staining medium). The cells were washed twice with staining medium and incubated on ice for 30 min with fluorescently labeled antibodies against CD45, CD34, or CD49b. CD45 staining was used to filter out peripheral blood leukocytes. After incubation with antibodies, the cells were washed three times and then incubated with 1 μg/mL propidium iodide (PI, Sigma-Aldrich, P4170) to label nonviable cells. The cells were then subjected to cell sorting by using a FACSAria cell sorter (BD Biosciences). Most cells were selected for analysis on the basis of forward vs. side scatter profile. Dead cells (PI+) and leukocytes (CD45+) were excluded by electronic gating. The remaining cells were analyzed for CD34 and CD49b expression to harvest $CD34^+/CD49b^+$, $CD34^+/CD49b^-$, and $CD34^-/CD49b^-$ populations.

RNA isolation and real-time quantitative PCR

Total RNA was isolated using the Qiagen Allprep RNA/DNA mini kit (Qiagen, 80204) or Qiagen RNeasy RNA micro kit (Qiagen, 74004). cDNA was synthesized using qScript cDNA SuperMix (total RNA >100 ng; VWR International, 95048–100) or SuperScript VILO Master Mix (total RNA <100 ng; Thermo Fisher Scientific, 11754050). mRNA levels of WNT4, FZD6, cyclin D1, and cMyc were quantified using real-time qPCR normalized to TATA-binding protein (TBP), as previously described (36). Each real-time qPCR assay was performed using cells isolated from at least three patient samples.

Cell counting Kit-8 (CCK8) proliferation assay

CCK-8 is commonly used to determine the number of live cells in cell proliferation assays. Dehydrogenase activities within live cells reduce the tetrazolium salt provided by the CCK8 reagent and produce a yellow-color formazan dye, which is directly proportional to the number of living cells. 5 X 10^3 primary (passage zero) LM cells were seeded in 96-well plates. Prior to WNT4 or MK-2206 treatment, cells were starved in phenol-red free DMEM/F12 medium containing 0.2% cFBS and 1% antibiotic/antimycotic for 24 hours. Cells were treated with WNT4 or MK-2206 for 96 hours prior to the CCK8 proliferation assay. 10 μl of CCK8 reagent was added to 100 μl media to start the CCK8 assay. Cells were incubated with CCK8 reagent for four hours and absorbance at 450nm was measured using a microplate reader. CCK8 assay was performed using cells isolated from at least three patient samples.

WNT4 siRNA knockdown

Primary LM cells (passage zero) were transfected with two different WNT4 siRNAs (Dharmacon, siWNT4–1: D-008659–04-0002 and siWNT4–2: D-008659–01-0002) or control scrambled siRNA (D-001810–10-05) using Dharmafect 1 (Dharmacon, Cat# T-2001–02) transfection reagent following the manufacturer protocol. The siRNA concentration in the cells was 25 nM. 72 hours after transfection, the cells were harvested for real time qPCR and western blot analyses. siRNA knockdown assay was performed using cells isolated from at least three patient samples.

Immunoblot analysis

Protein was extracted from LM primary cells using radioimmunoprecipitation assay buffer, followed by quantification using bicinchoninic acid protein assay reagent (ThermoFisher Scientific, 23225) per the manufacturer's protocol. Total protein was diluted in reducing 4X LDS sample buffer (ThermoFisher Scientific, NP0007), electrophoresed on a 4% to 12% Novex Bis-Tris polyacrylamide precast gel (ThermoFisher Scientific, NP0321BOX), and transferred onto polyvinylidene difluoride membrane. Incubation with primary antibodies (Supplementary Table 1) was performed at 4°C in 5% nonfat milk overnight. The membranes were then washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for one hour at room temperature. Detection was performed using Luminata Crescendo horseradish peroxidase substrate (Millipore, WBLUR0100). CK1 phosphorylates β-catenin at Ser 45, which primes β-catenin for subsequent phosphorylation by GSK-3β (37–39). The level of non-phosphorylated β-catenin at Ser 45 has been used as an indicator of active β-catenin. Phosphorylation of β-catenin at Ser 33, Ser 37, and Thr 41 are indications of β-catenin degradation targeted by GSK-3β; while its phosphorylation at Ser 552, mostly by AKT, promotes its transcriptional activity (32, 37, 38). To evaluate the effect of WNT4 on β-catenin and AKT pathway activation, we examined the levels of non-phosphorylated at Ser 45 and phosphorylated at Ser552 β-catenin and its downstream targets, Axin2, cyclin D1, and c-Myc. We quantified each Western Blot using Image J (version 2.0.0) and normalized the target protein levels to the β-actin levels. Immunoblot analysis was performed using samples from at least three patients.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 (GraphPad Inc.); detailed statistical test descriptions are reported in each figure legend. Illumina Human HT-12 microarray data published by Moravek, et al was re-analyzed using *lumi* and *limma* R packages (12). No sample was excluded from the analysis. Paired Student's t-tests were performed to compare means between two treatment groups; One-way ANOVA followed by pairwise comparison analyses were performed to compare means among three or more treatment groups; Two-way ANOVA followed by pairwise comparison analyses were performed to compare among treatment groups with two independent variables and investigate the interaction between the two variables. Values were considered statistically significant when $P<0.05$. All experiments were repeated with samples from at least three patients, with the patient number (n) noted in the figure legends. Data points in the bar plots represent biological replicates from different patients and error bars represent SEM.

Results

WNT/β-catenin signaling components are differentially expressed among the three LM cell populations

Due to the importance of the WNT/β-catenin signaling pathway in LM, we first evaluated β-catenin activity by examining the protein levels of phosphorylated β-catenin and its downstream transcriptional target, Axin2 in each LM cell population. The level of non-phosphorylated β-catenin at Ser 45 has been used as an indicator of active β-catenin; while its phosphorylation at Ser 552, mostly by AKT, promotes its transcriptional activity (32, 37, 38). Using immunoblots, we found that LSC harbored the highest levels of non-phosphorylated Ser 45, phosphorylated Ser 552, and total β-catenin among the three LM cell populations (Figures 1A and 1B). Consistently, LSC also had the highest level of Axin2, indicating β-catenin signaling activation in the LSC population (Figures 1A and 1B).

We then examined the mRNA levels of WNTs and their receptors (Frizzles and LRPs) in the three LM cell populations using previously published microarray data (12). We found that several WNT ligands and receptors were differentially expressed among the three LM cell populations, including WNT4, WNT2, FZD6, and FZD4 (Figure 1C). On average, WNT ligand levels were higher in LIC, while their receptors were higher in LSC, and WNT4 was one of the most highly expressed WNT ligands in the LIC population. We further validated that the highest expression of WNT4 was in LIC and its receptor FZD6 in LSC using freshly sorted LM cell populations from independent LM tissues (n = 4, Figure 1D).

WNT4 drives AKT-dependent β-catenin activation in LM cells

Focusing on the function of WNT4 in LM, we treated total primary LM cells with vehicle (0.1% BSA in PBS) or WNT4 (200 ng/ml) for different time lengths. Immunoblot analysis showed that short term WNT4 treatment did not influence the level of non-phosphorylated β-catenin at Ser 45, rather it specifically increased β-catenin phosphorylation at Ser 552 as early as 15 minutes, and the induction lasted around an hour (Figures 2A and 2B). Since AKT phosphorylates β-catenin at Ser 552, we also examined the effect of WNT4 on AKT phosphorylation. AKT phosphorylation robustly increased after 15 minutes of WNT4

treatment, after which the magnitude of stimulation gradually decreased and disappeared by 120 minutes, suggesting that WNT4 function might be AKT-dependent in LM.

We further investigated whether AKT activation is sufficient for β-catenin phosphorylation at Ser 552. Primary LM cells were treated with the AKT activator, SC79 (10 μM) for different lengths of time. SC79 treatment enhanced AKT phosphorylation after 30 minutes treatment and reached significance after 1h (Figures 2C and 2D). As expected, β-catenin phosphorylation at Ser 552 increased at 30 minutes and peaked at two hours post-treatment, which was accompanied by increased levels of active β-catenin (non-phosphorylated β-catenin at Ser 45) and its downstream target, Axin2 (Figures 2C and 2D). To explore whether WNT4 signal is mediated by AKT pathway, we then pretreated LM primary cells with or without the AKT inhibitor, MK-2206 for 24 hours, followed by treatment with WNT4 for 30 minutes. Treatment with WNT4 for 30 minutes dramatically upregulated the protein levels of phosphorylated β-catenin (Ser 552) and AKT, and this effect was blocked by pre-treatment with MK-2206 (Figures 2E and 2F). These findings suggested that WNT4 could activate β-catenin by inducing an AKT-mediated β-catenin phosphorylation at Ser 552 in primary LM cells.

WNT4 stimulates primary LM cell proliferation

To determine whether WNT4 regulates LM cell growth, we treated primary LM cells with different concentrations of WNT4 for 96 hours and quantified cell proliferation using the CCK8 assay. WNT4 treatment increased LM cell proliferation in a dose-dependent manner, with changes from baseline, reaching statistical significance at 200 and 400 ng/ml WNT4 (Figure 3A). Under the same treatment conditions, WNT4 significantly increased c-Myc protein level 120 min after treatment and cyclin D1 protein levels 360 minutes after treatment (Figures 3B and 3C), lagging behind the induction of AKT and β-catenin activation (Figures 2A and 2B). Consistent with the protein level changes, WNT4 treatment for one hour also significantly upregulated c-Myc and cyclin D1 mRNA levels (Figure 3D). To further confirm that these effects were caused by WNT4, we performed siRNA knockdown using two different siRNAs (Figures 3E, siWNT4–1 and siWNT4–2). siWNT4–1 and siWNT4–2 both consistently reduced protein levels of the proliferating cell nuclear antigen (PCNA, a cell proliferation marker) in LM primary cells, however, c-Myc and cyclin D1 protein levels were only significantly decreased by siWNT4–2 (Figures 3F and 3G).

AKT inhibitor, MK-2206 blocks WNT4-induced LM cell proliferation

Because MK-2206 efficiently blocked AKT-induced β-catenin activation (Figures 2E and 2F), we further evaluated its effect on WNT4-mediated cell proliferation. WNT4-induced upregulation of c-Myc, cyclin D1, and PCNA mRNA and protein levels was completely abrogated by pretreatment with MK-2206 (Figures 4A–4C). We also measured the proliferation ability following 96 hours of treatment with vehicle or WNT4 in the presence or absence of MK-2206 pretreatment. The number of live cells was 2-fold higher after WNT4 treatment compared to vehicle treatment, an effect that was blocked by MK-2206 pretreatment (Figure 4D).

LSC had the highest level of FZD6 receptor among the three LM cell populations (Figure 1D); therefore, we investigated whether WNT4 regulates LSC function. We treated freshly FACS-sorted LM cell populations with WNT4 for one hour. WNT4 treatment upregulated mRNA levels of the pro-proliferative genes, c-Myc and cyclin D1 specifically in LSC, and pretreatment with MK-2206 abolished these effects (Figure 4E), indicating that WNT4 may stimulate LSC proliferation via activating AKT signaling pathway in these cells.

Discussion

In this study, we showed that WNT/β-catenin signaling pathway components are distinctly expressed among the three LM cell populations: WNT4 was predominantly expressed in LIC, while FZD6 was primarily expressed in LSC. WNT4 stimulated phosphorylation of AKT and induced β-catenin activation in primary LM cells, leading to increased proliferation. Importantly, WNT4 upregulated the expression of pro-proliferative genes, c-Myc and cyclin D1, specifically in LSC. These WNT4-induced effects were completely abolished by the AKT inhibitor, MK-2206. Thus, we uncovered novel WNT4-mediated interplay between the AKT and β-catenin pathways, which may contribute to LSC proliferation and LM growth.

The PI3K/AKT pathway regulates various cellular processes, such as proliferation, growth, apoptosis, and cytoskeletal rearrangement (22). Studies have demonstrated important roles for AKT in LM pathogenesis. Kovacs et al. reported the presence of abundant phosphorylated AKT in LM (25), and treatment of LM cells with promegestone activates the AKT pathway, which stimulates proliferation and inhibits apoptosis of LM cells (25, 30). AKT inhibition by MK-2206 was shown to promote caspase-independent cell death and inhibit LM growth (29). Here, we observed that WNT4 also stimulates AKT phosphorylation in LM cells, suggesting that this pathway may contribute to dysregulated AKT activity in LM. Importantly, WNT4 induced the expression of pro-proliferative genes c-Myc and cyclin D1 specifically in LSC. Our current findings suggest a crucial role of AKT in LM tumorigenesis through stem cell regulation and provide mechanistic evidence to support therapeutic targeting of the AKT pathway in LM.

Our finding that the β-catenin signaling activity is upregulated in the LSC population and that a specific WNT ligand, WNT4, is responsible for the paracrine interaction among the LM cell populations supports previous work suggesting that the WNT/β-catenin pathway promotes LM tumorigenesis through regulation of LM stem cells (20, 40, 41). We found that the AKT pathway is activated by WNT4 in LM and is indispensable for sustaining the activation of the β-catenin pathway through phosphorylation of β-catenin at Ser 552. Studies have suggested that WNT1 activates Dishevelled, which interacts with integrin-linked kinase, leading to AKT phosphorylation and activation (33–35). However, it remains unclear how AKT is activated by WNT4 in LM. Interestingly, we also observed that AKT activator increased the level of non-P-β-catenin at Ser 45. CK1 has been identified as a regulator of β-catenin stability and activity (42, 43), and its activity can be regulated by post-translational modifications, mainly by reversible phosphorylation either through autophosphorylation or site-specific phosphorylation mediated by cellular kinases, such as PKA and AKT (44, 45). Thus, AKT may influence β-catenin phosphorylation at Ser 45 by regulating CK1 activity in

LM primary cells. Given the critical roles of AKT and WNT/β-catenin in LM tumorigenesis, the mechanisms underlying WNT4-activated AKT pathway warrant further investigation.

It is worth noting that LIC and LDC also expressed some Frizzled receptors such as FZD7 (Figure 1C), however, WNT4 treatment did not affect cyclin D1 and c-Myc expression in these cells, at least under the *in vitro* treatment conditions used in this study (Figure 4E). This is consistent with previous observation that, when cocultured with myometrial smooth muscle cells, LM side population (LM stem) cells but not main population (differentiated LM) cells, proliferate in response to WNT ligands secreted by myometrial smooth muscle cells (20). Lyons et al reported that WNT4 has a high potency towards FZD6 to activate the β-catenin canonical pathway, suggesting that WNT4/FZD6 signaling might be important for the activation of β-catenin in LSC (46). Thus, we reasoned that the response of LSC primarily accounts for the gene expression changes induced by WNT4 treatment in cultured total LM primary cells (Figures 3D and 4E).

Previous research has demonstrated that WNT4 expression is stimulated by mutant MED12 and upregulated in LM carrying the MED12 mutation, especially in LM with the most common MED12 mutation, G44D (18, 47). Although detailed studies are needed to investigate the mechanisms by which WNT4 is upregulated in this particular LM subtype, this study help us better understand subtype-specific LM phenotypes and pave the way for personalized therapeutic strategies for this devastating disease.

Conclusions

In summary, we discovered that WNT4 induces AKT-dependent β-catenin activation and cell proliferation in LM cells and is, therefore, a novel regulator of uterine LM stem cell function. Our study suggests that interfering with WNT-AKT axis-regulated LM stem cell proliferation may be a potentially effective strategy to reduce the growth of existing LM or prevent the growth of new tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Capsule

We discovered that WNT4 regulates leiomyoma cell proliferation, especially the stem cells, via AKT activation, which bettered the understanding of leiomyoma stem cell regulation and revealed potential therapeutic targets.

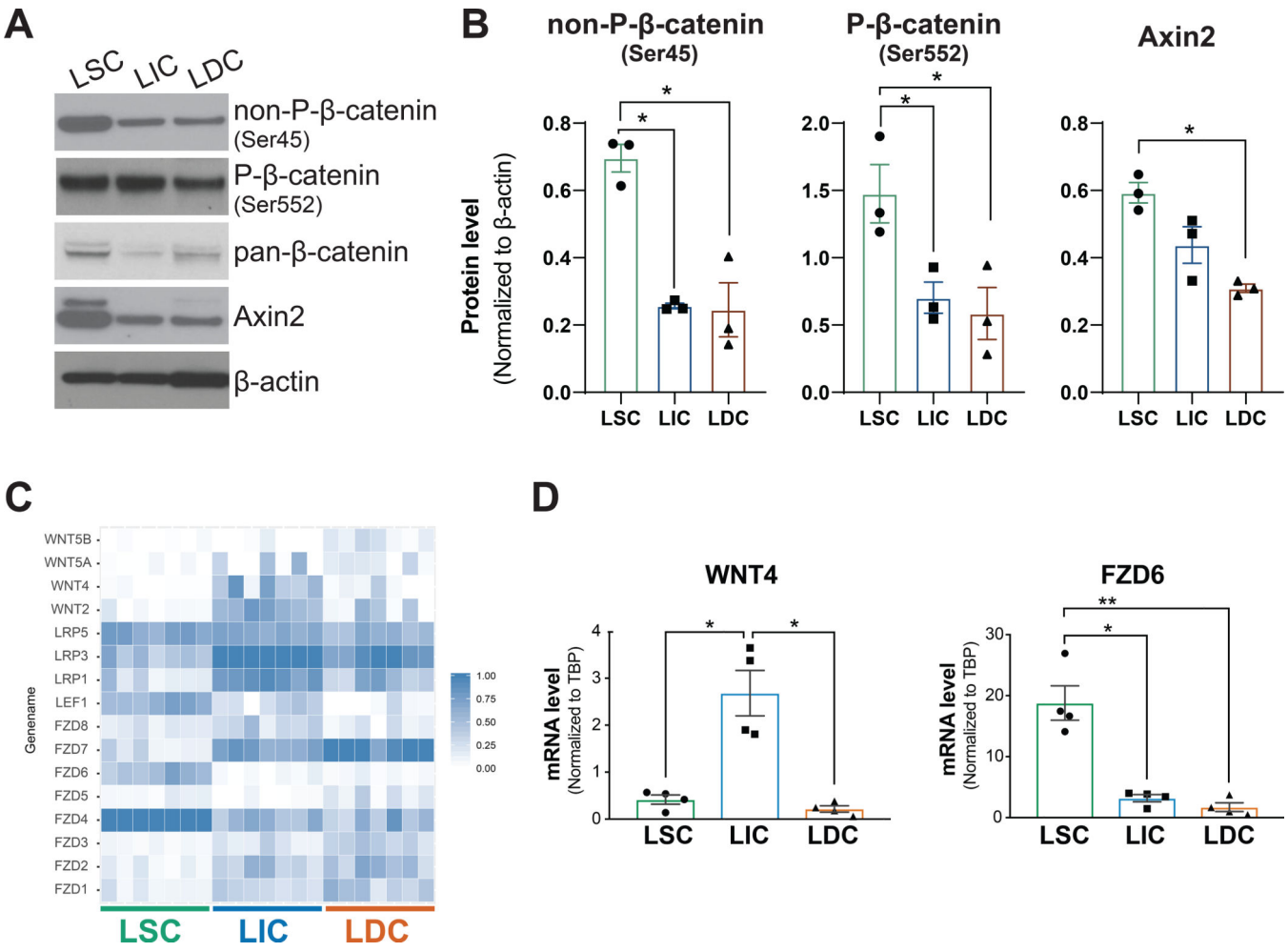

Figure 1. WNT/β-catenin pathway components are differentially expressed in the leiomyoma (LM) cell populations.

A) and **B**) showing the representative immunoblots and quantification, respectively, of the β-catenin phosphorylation and Axin2 protein levels in freshly isolated LM cell populations (mean ± SEM, **P*<0.05, *n* = 3, one-way ANOVA, LDC: LM differentiated cells, LIC: LM intermediate cells, LSC: LM stem cells). **C**) Heatmap showing the expression levels of WNTs and their receptors (Frizzles and LRPs) in the three LM cell populations from the Illumina Human HT-12 microarray data (*n* = 7). **D**) Real time qPCR validation of mRNA levels of WNT4 and its receptor, FZD6 in the LM cell populations (mean ± SEM, **P*<0.05, ***P*<0.01, *n* = 4, one-way ANOVA).

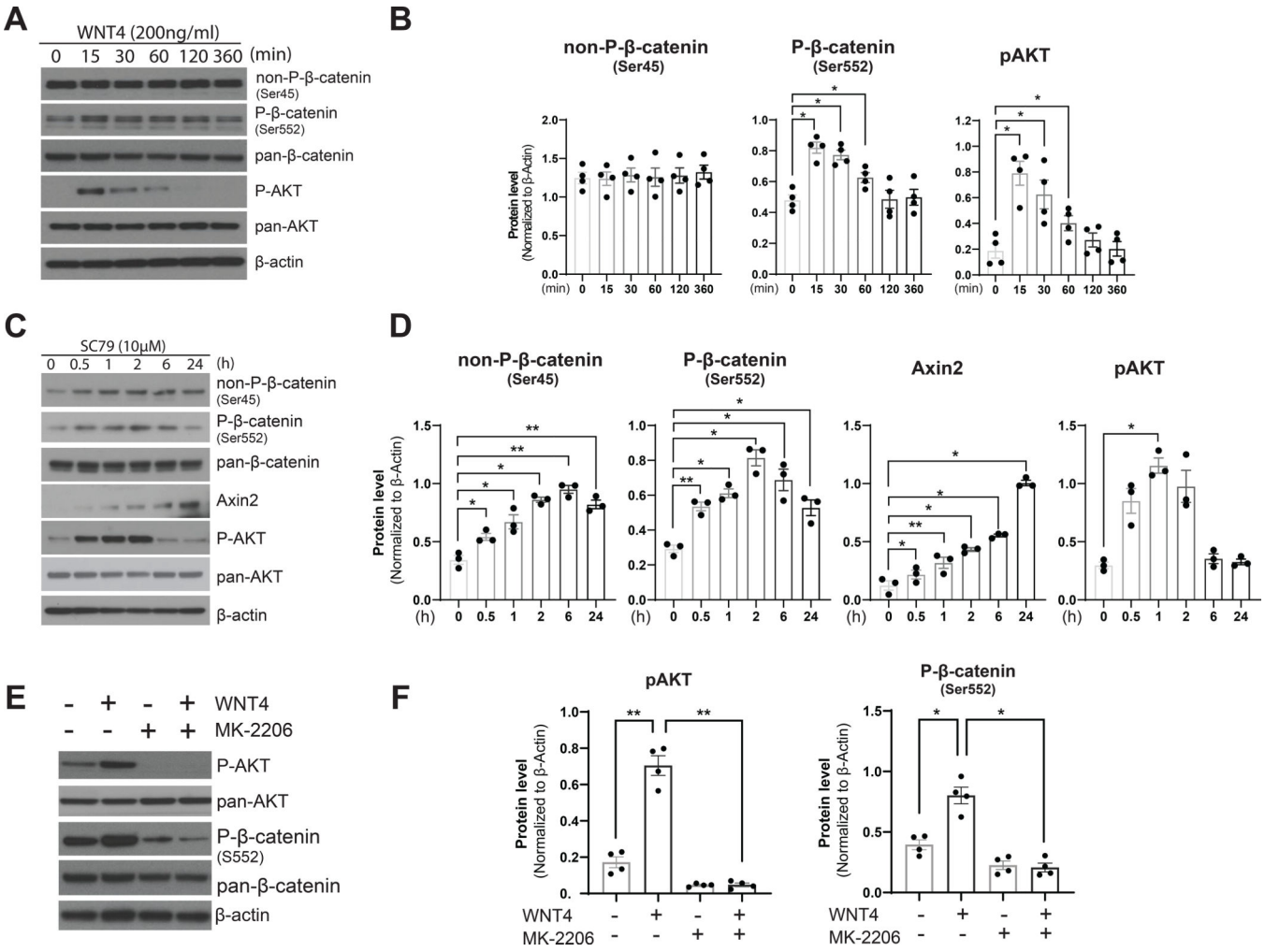

Figure 2. WNT4 drives AKT-dependent β-catenin activation in LM cells.

A) and **B**) showing the representative immunoblots and quantification, respectively, of P-AKT, P-β-catenin (Ser 552), and non-β-catenin (Ser 45) after WNT4 treatment (200 ng/ml) over time in primary LM cells (mean ± SEM, **P*<0.05, *n* = 4, one-way ANOVA). **C**) and **D**) showing the representative immunoblots and quantification, respectively, of P-AKT, P-β-catenin (Ser 552), non-β-catenin (Ser 45), and Axin2 protein levels in primary LM cells treated with AKT activator, SC79 (10 μM) over time (mean ± SEM, **P*<0.05, ***P*<0.01, *n* = 3, one-way ANOVA). **E**) and **F**) showing the representative immunoblots and quantification, respectively, of changes in AKT and β-catenin phosphorylation levels in LM primary cells treated with or without MK-2206 (1 μM) for 24 hours. Cells were stimulated with vehicle (0.1% BSA in PBS) or WNT4 (200 ng/ml) for 30 minutes prior to harvest (mean ± SEM, **P*<0.05, ***P*<0.01, *n* = 4, two-way ANOVA).

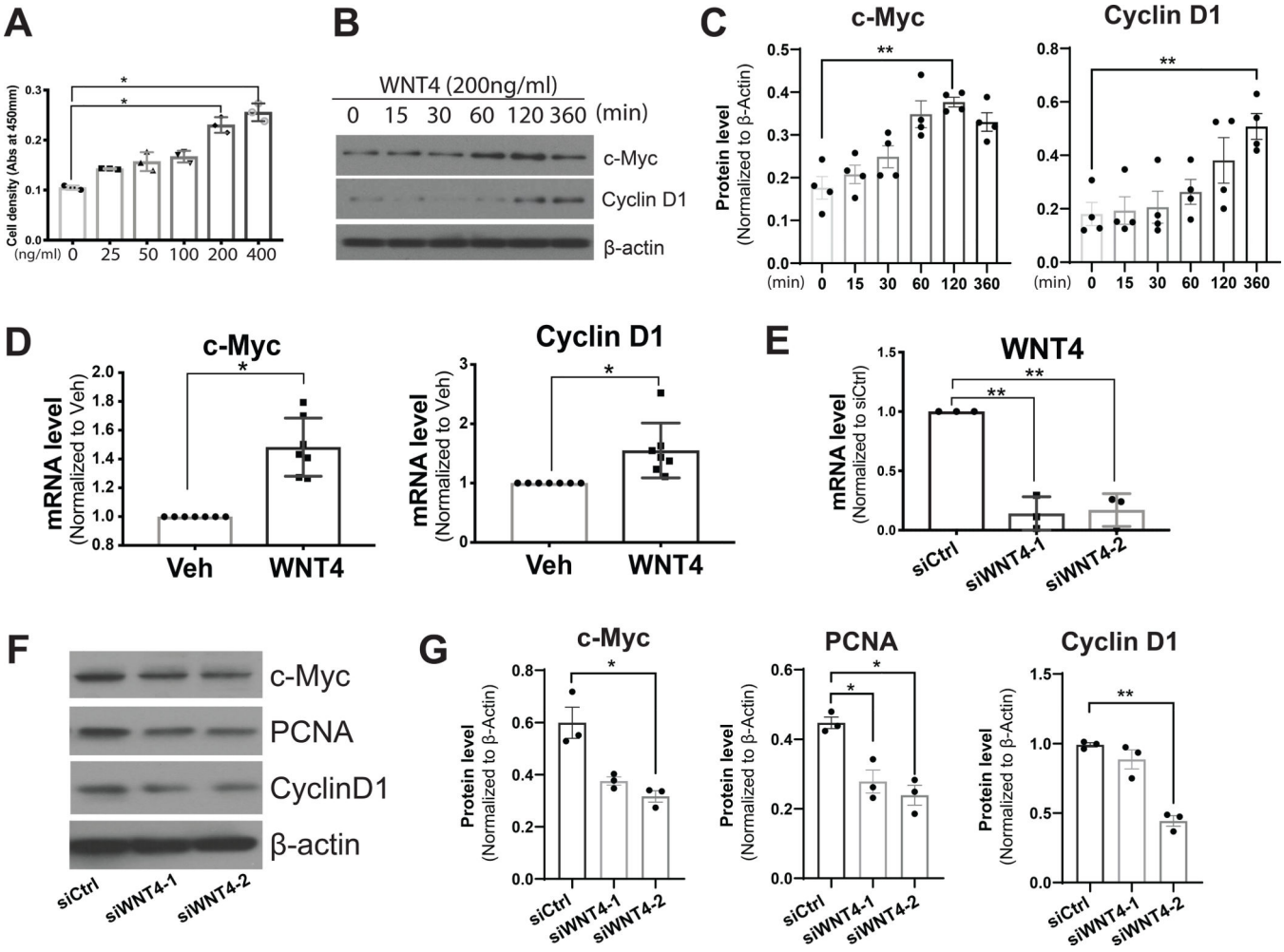

Figure 3. WNT4 stimulates primary LM cell proliferation.

A) Cell density of primary LM cells treated with vehicle (0.1% BSA in PBS) or WNT4 at various concentrations for 96 hours (mean ± SEM, $n = 3$, $^{*}P<0.05$, one-way ANOVA). **B**) and **C**) showing the representative immunoblots and quantification, respectively, of the protein levels of the pro-proliferative genes (c-Myc and cyclin D1) after WNT4 treatment (200 ng/ml) for the indicated time length (mean ± SEM, $^{**}P<0.01$, $n = 4$, one-way ANOVA). **D**) mRNA levels of c-Myc (left) and cyclin D1 (right) in LM primary cells treated with vehicle (0.1% BSA in PBS) or WNT4 (200 ng/ml) for one hour (mean ± SEM, $n = 7$, $^{*}P<0.05$, paired t-test). **E**) *WNT4* mRNA levels in LM cells 72 hours post-transfection with *WNT4* siRNAs (siWNT4–1 and siWNT4–2) or scrambled control siRNA (siCtrl; mean ± SEM, $n = 3$, $^{**}P<0.01$, paired t-test). **F**) and **G**) showing the representative immunoblots and quantification, respectively, of the protein levels of the pro-proliferation genes (c-Myc, PCNA, cyclin D1) 72 hours post-transfection with *WNT4* siRNAs or control siRNA (mean ± SEM, $^{*}P<0.05$, $^{**}P<0.01$, $n = 3$, one-way ANOVA).

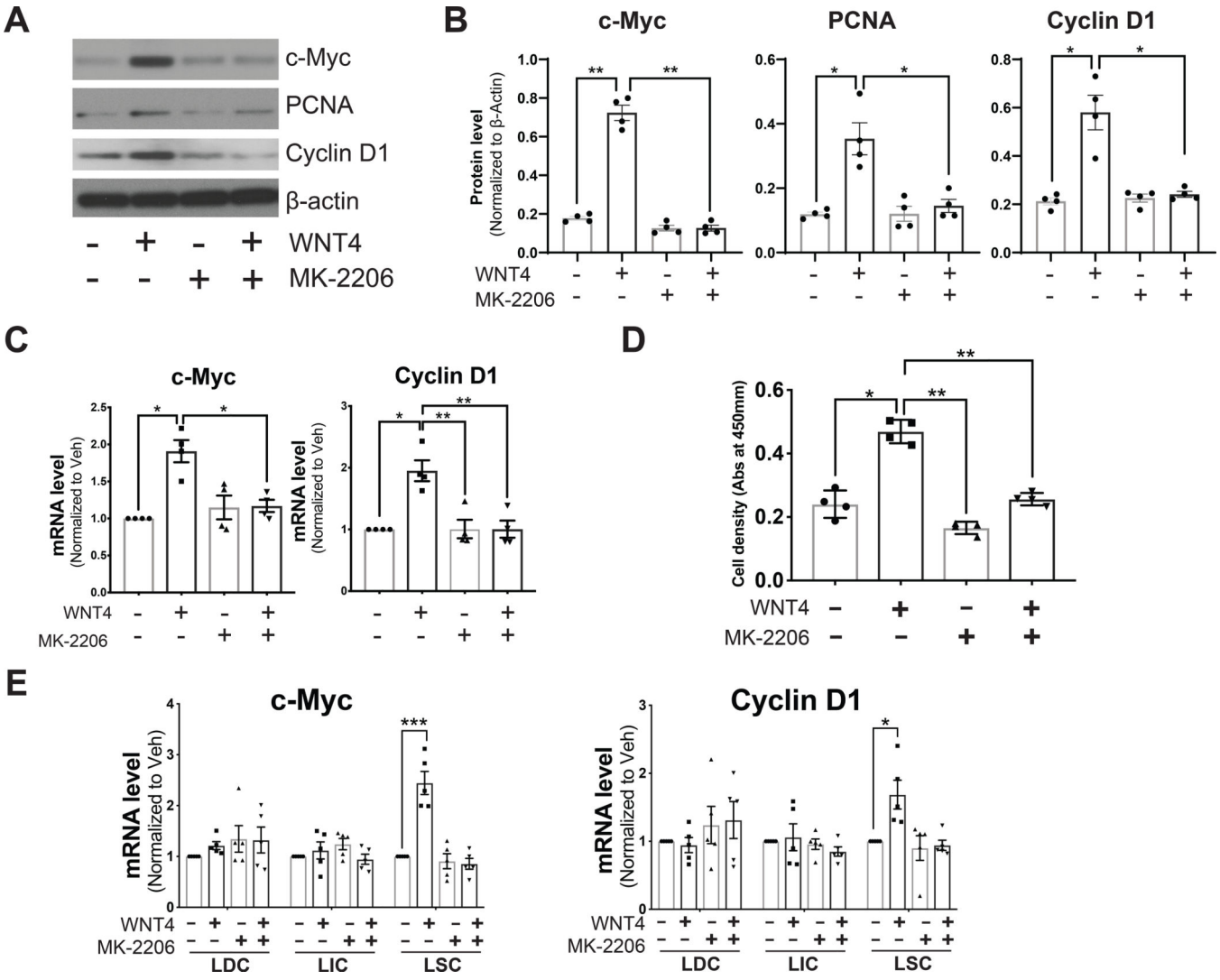

Figure 4. AKT inhibitor MK-2206A blocks WNT4-induced LM cell proliferation.

A) and **B**) showing the representative immunoblots and quantification, respectively, of changes in protein levels of phosphorylated AKT and proliferation-associated genes (c-Myc, PCNA and cyclin D1) in LM primary cells treated with or without AKT inhibitor, MK-2206 (1 μM) for 48 hours; treatments were refreshed every 24 hours. Cells were stimulated with vehicle or WNT4 (200 ng/ml) 24 hours prior to harvest (mean ± SEM, **P*<0.05, ***P*<0.01, *n* = 4, two-way ANOVA). **C**) mRNA levels of c-Myc (left) and cyclin D1 (right) in LM primary cells treated with or without MK-2206 for 24 hours; cells were stimulated with vehicle or WNT4 (200 ng/ml) one hour prior to harvest (mean ± SEM, *n* = 4, **P*<0.05, ***P*<0.01, two-way ANOVA). **D**) Density of LM primary cells treated with or without WNT4 (200 ng/ml) in the presence or absence of AKT inhibitor MK-2206 (1 μM) for 96 hours (mean ± SEM, *n* = 4, **P*<0.05, ***P*<0.01, two-way ANOVA). **E**) mRNA levels of c-Myc (left) and cyclin D1 (right) in individual LM cell populations treated with or without AKT inhibitor, MK-2206 (1 μM) for 24 hours; cells were stimulated with vehicle or WNT4

(200 ng/ml) one hour prior to harvest (mean ± SEM, $n = 5$, *$P<0.05$, ***$P<0.001$, two-way ANOVA).