

Progesterone initiates Wnt- β -catenin signaling but estradiol is required for nuclear activation and synchronous proliferation of rat uterine stromal cells

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Abstract

Progesterone pretreatment of ovariectomized rat uteri increases the number of synchronously proliferating stromal cells in response to estradiol 17- β . To identify the signals involved in stimulating synchronous proliferation, sexually mature ovariectomized rats were injected with progesterone (2 mg) for 3 consecutive days. Estradiol 17- β (0.2 μ g) was administered to initiate cell cycle entry. Uterine samples were removed at various times after hormone administration and changes in wingless (Wnt) pathway effectors and gene targets were identified by microarray. Progesterone pretreatment decreased glycogen synthase kinase-3 β (GSK-3 β) and increased expression of T-cell factor/lymphoid enhancer factor (TCF/LEF). GSK-3 β protein decreased markedly in the uterine stroma of progesterone-pretreated uteri with the concomitant appearance of β -catenin in these stromal cells. Translocation of β -catenin from the cytosol to the nuclei in progesterone-pretreated

stromal cells was stimulated in response to estradiol. β -Catenin binding to TCF/LEF increased ($P < 0.05$) in progesterone-pretreated uteri in response to estradiol. Progesterone stimulated the expression of the Wnt target gene urokinase plasminogen activator receptor (uPA-R) in the periluminal uterine stromal cells. The expression of uPA-R increased in progesterone-pretreated stromal cells in response to estradiol administration. Together, the results indicate that progesterone initiates Wnt signaling in the uterine stroma by down-regulating GSK-3 β . However, nuclear translocation of β -catenin and sufficient complex formation with TCF/LEF to activate stromal cell cycle entry requires estradiol. Stimulation of a uterine stromal cell line to proliferate and differentiate resulted in β -catenin accumulation, suggesting that endocrine-dependent Wnt signaling controls proliferation and differentiation (decidualization).

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Introduction

Female sex steroids control proliferation and differentiation of target cells by altering the rates of specific gene transcription (Tsai & O'Malley 1994, Hall *et al.* 2001, Li & O'Malley 2003). In the endometrium of the rat (Rider & Psychoyos 1994) and the mouse (Finn & Martin 1967), estradiol 17- β stimulates proliferation of epithelial cells. At day 4 of pregnancy in the rat, there is a proliferative switch from epithelial to stromal compartments (Rider & Psychoyos 1994). This proliferative switch is dependent upon progesterone because administration of progesterone receptor antagonists to mice (Cullingford & Pollard 1988) and rats (Rider & Psychoyos 1994) blocks stromal cell proliferation. Proliferating stromal cells differentiate to form the maternal interface with the placenta and comprise the tissue referred to as the decidua (Bell 1983). Decidualization is required for successful reproduction because mice lacking genes that

control stromal cell differentiation cannot maintain pregnancy (Lydon *et al.* 1995, Robb *et al.* 1998, Yao *et al.* 2003).

Glycogen synthase kinase 3 (GSK-3) is a multifunctional protein that has been identified as two highly homologous variants (GSK-3 α /GSK-3 β) in eukaryotic cells (Hoefflich *et al.* 2000, Frame & Cohen 2001, Doble & Woodgett 2003). Inactivation of GSK-3 β stimulates cellular proliferation and differentiation through two separate mechanisms. First, GSK-3 β is normally active in cells where it phosphorylates cyclin D1 which inhibits nuclear localization of the cyclin and blocks cell cycle entry (Diehl *et al.* 1998). In response to mitogenic agents, GSK-3 β is inactivated by phosphorylation and cyclin D1 is retained in the nucleus where it stimulates G1 transit (Diehl *et al.* 1998). Secondly, GSK-3 β is one component of a multimeric complex containing several proteins including axin, adenomatous polyposis coli (APC), and β -catenin. Activation of the wingless (Wnt) signal transduction pathway inactivates GSK-3 β by mechanisms

that are not fully understood and stimulates the cytoplasmic accumulation of β -catenin. As the amount of β -catenin in the cytoplasm increases, the protein translocates to the nucleus where it binds with TCF/LEF (T-cell factor 1/lymphoid enhancing factor-1) and co-regulates Wnt target genes (Van de Wetering *et al.* 1996, Polakis 2000, Knapp *et al.* 2001, Hurlstone & Clevers 2002, Bienz 2005, Daniels & Weis 2005, Reva & Clevers 2005).

Wnt signal transduction plays a key role in the early development of most organisms (Nusse 2005, Tian *et al.* 2005, Widelitz 2005). In the female reproductive tract of mammals, Wnt4 mutants fail to form Mullerian ducts and die at birth (Vainio *et al.* 1999). Wnt7a mutants are viable but exhibit malformations in the uterus (Parr & McMahon 1998). Wnt receptors have been localized to the maternal decidua (Fujita *et al.* 2002) and epithelium in response to estrogen (Hou *et al.* 2004). Inhibition of Wnt signal transduction prevents implantation in the mouse (Mohamed *et al.* 2005), and down-regulates estrogen-dependent β -catenin expression (Hou *et al.* 2004).

Progesterone pretreatment of ovariectomized rat uteri increases the number of synchronously proliferating uterine stromal cells in response to estradiol (Tachi *et al.* 1972, Rider & Psychoyos 1994). Progesterone pretreatment blocks the estrogen-dependent nuclear localization of cyclin D1 in the uterine epithelium but not in the stroma (Rider *et al.* 2003). We postulated that the cell-specific nuclear localization of cyclin D1 in the uterine stroma occurred by a mechanism that inactivates GSK-3 β and allows progesterone-pretreated stromal cells to enter the cell cycle in response to estradiol. In the present study, we have used this model system to gain insight into the mechanism(s) by which progesterone pretreatment synchronizes stromal cell proliferation. We were also interested in identifying the molecular basis for estradiol action that is necessary to stimulate progesterone-pretreated stromal cells to enter the cell cycle. The results show that the control of Wnt signaling by sex hormones in the uterine stroma is a two-step process. Progesterone is necessary to down-regulate GSK-3 β expression leading to the accumulation of β -catenin in the stromal cytoplasm. However, sufficient translocation of β -catenin and complex formation with TCF/LEF for cell cycle entry requires estradiol.

Materials and Methods

Animals and hormone treatments

Sexually mature (150–175 g body weight) Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were bilaterally ovariectomized (OVX) and rested for 10 days. Rats were housed under a 14 h light:10 h darkness cycle at Pittsburg State University and provided with rodent chow and water available *ad libitum*. Animals were treated in accordance with the principles and the procedures outlined by the National Institutes of Health (NIH) Guidelines for the Care and Use of Experimental Animals. The Pittsburg State University Animal

Care Committee approved protocols for the care and the use of rats. To stimulate stromal cell proliferation, OVX rats were injected subcutaneously with progesterone (2 mg; Sigma–Aldrich) dissolved in sesame oil daily for 3 consecutive days. On the fourth day, estradiol 17- β (0.2 μ g; Sigma–Aldrich) was injected subcutaneously. This hormone regimen increases the number of synchronously proliferating stromal cells three- to fivefold compared with normally pregnant animals (Rider & Psychoyos 1994). The uterine horns were removed at 6 and 12 h post-estradiol injection when a significant number of stromal cells are in S and M phases of the cell cycle (Rider *et al.* 2003).

Indirect immunoperoxidase analysis

Uterine tissue was removed under anesthesia and the uterine horns from OVX rats, those pretreated with progesterone for 72 h (0 hE), and those pretreated with progesterone and estradiol for 6 and 12 h (6 hE, 12 hE) were fixed in 4% paraformaldehyde and embedded in paraffin using methods standard in our laboratory (Rider & Psychoyos 1994). Sections (~8 μ m) were cut on a microtome and placed on Superfrost Plus slides (Fisher Scientific, Hanover Park, IL, USA). Sections were treated with 10 mM sodium citrate at 95 °C for 5 min to unmask the antigens. To remove endogenous peroxidase activity, tissue sections were quenched in 0.3% hydrogen peroxide (Sigma–Aldrich) in methanol at 22 °C for 30 min. Samples were blocked for 18 h in a blocking buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.2% gelatin, 0.05% Tween 20, 0.5% (w/v) powdered milk) at 4 °C. The slides were washed in PBS and reacted with GSK-3 β (2 μ g/ml, SC-7291, Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-GSK-3 β (2 μ g/ml; 9336, Cell Signaling Technology, Danvers, MA, USA), β -catenin (2 μ g/ml; SC-1496, Santa Cruz Biotechnology), and urokinase plasminogen activator receptor (uPA-R, 2 μ g/ml; SC-10815, Santa Cruz Biotechnology) antibodies for 18 h at 4 °C. To evaluate specificity of the reaction, some sections were incubated without primary antibody. Sections were reacted with biotinylated affinity purified anti-mouse (GSK-3 β , phospho-GSK-3 β) and anti-rabbit (β -catenin, uPA-R) secondary antibodies (Vector Laboratories, Burlingame, CA, USA) for 30 min at 22 °C. Slides were exposed to the Vectastain ABC reagent (Vector Laboratories), washed in PBS, and reacted for 2 min with equal volumes of 1 μ g/ml diaminobenzidine (Aldrich, Milwaukee, WI, USA) dissolved in 0.1 M Tris–HCl (pH 7.2) and 0.1% (v/v) hydrogen peroxide diluted in PBS. Slides were counter stained with 1% methyl green dye in deionized water. The uterine horns from at least three separate animals at each time point were examined. Representative sections were photographed using an Olympus BX41 microscope equipped with a digital camera.

Western blots

The uterine horns were removed and pooled from OVX rats ($n=5$ per experiment) without further treatment. Some OVX rats ($n=5$ per experiment) were treated with

progesterone (2 mg) for 3 consecutive days. The uterine horns were removed and pooled without further treatment (0 hE). A separate group of OVX rats ($n=5$ per treatment) were pretreated with progesterone for 3 days. On the fourth day, estradiol (0.2 μg) was injected subcutaneously and the uterine horns were removed at 6 (6 hE) and 12 (12 hE) h without further treatment. Total uterine proteins were obtained by homogenization of the uterine horns in five volumes of homogenization buffer containing protease inhibitor cocktails as described previously (Jones *et al.* 2000). Uterine stromal cell extracts were prepared from uterine stromal cell line, isolation III (UIII) as described previously (Jones *et al.* 2000). Extracts (500 μl) were incubated with β -catenin and β -actin antibodies (2 μl antibody per 100 μl extract) for 18 h at 4 °C. The antigen–antibody complexes were collected by immunoprecipitation. Samples were centrifuged and washed thrice in PBS containing 0.1 M NaCl. The samples were heated at 95 °C for 3 min in SDS-sample buffer, cooled to 22 °C and the proteins were size-fractionated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane using standard methods (Jones *et al.* 2000, Rider *et al.* 2003).

For alkaline phosphatase detection, the membranes were reacted with primary antibody (β -catenin, 1:100 dilution) for 90 min at 22 °C. The blots were washed and reacted with species-specific alkaline phosphatase secondary antibody diluted in the ratio of 1:1000. After washing, bound antibody was detected by incubating the blots with 0.3 mg/ml nitroblue tetrazolium and 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M NaHCO_3 , 1.0 mM MgCl_2 , pH 9.8. The size of the reactive protein was determined from prestained molecular size standards (Bio-Rad Laboratories, Inc.) and the relative amount of β -catenin was quantified by scanning densitometry of triplicate samples from three independent assays (Scion Image software from NIH).

For the chemiluminescent detection, the nitrocellulose membranes were reacted with a LEF-1 antibody (1 $\mu\text{g}/\text{ml}$, Upstate, Lake Placid, NY, USA) at 22 °C for 60 min. Some samples were treated identically except the membranes were reacted without the primary antibody (data not shown). The membranes were washed and incubated for 60 min with a species-specific horseradish peroxidase conjugated secondary antibody (1:50 000, Pierce Biotechnology, Rockford, IL, USA). The blots were incubated with a SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) for 5 min. The blots were exposed to X-ray film for 30–60 s to visualize chemiluminescent proteins. The size of the reactive protein was determined from prestained molecular size standards (Bio-Rad). The blots were stripped and reacted with an β -actin antibody (AC-15, Sigma) to control for equal protein loading. The relative amount of LEF-1 was quantified from triplicate samples on the same membrane by scanning densitometry using Scion Image software from NIH.

Microarrays

Uteri were excised from OVX rats ($n=6$). Three of these OVX rats were injected subcutaneously with progesterone (2 mg/daily) for 3 consecutive days (0 hE). The uterine horns were homogenized and total RNA was isolated from each pool (3OVX, 3OVX plus progesterone) using a single-step guanidine method (Chomczynski & Sacchi 1987). The RNA was converted to biotin-labeled cDNA from which biotin-labeled cRNA was generated using the standard methods (Yamamoto *et al.* 2005). Rat high-density oligonucleotide arrays representing 15 000 genes were supplied by Affymetrix (Cat no. 900404, Santa Clara, CA, USA). The biotin-labeled cRNAs were subjected to hybridization analysis at the DNA analysis core laboratory, Veterinary Medical Sciences, The University of Tokyo, Japan. Individual mRNA levels were scanned and scored for those mRNAs where the computer algorithm (Affymetrix) returned a 'present' call. Additional internal controls were carried out using the hybridization of the predetermined amount of cRNA to the microarray: BioB (1.5 pM), BioC (5 pM), Bio D (25 pM), and CreX (100 pM). Within the range, analyzed mRNA levels were linear ($y=0.026x-6.26$, $r^2=0.964$). Expression analysis of microarray experiments was performed using GeneSpring 7 (Silicon Genetics, Redwood City, CA, USA). The raw expression values were normalized to the 50th percentile per chip and to the median per gene. Only genes marked with a present flag in at least four out of the nine samples and a raw expression value of 50 or higher in at least four out of the nine samples were further considered in this study. The ratio of normalized expression levels for progesterone/OVX was calculated. The genes involved in the Wnt signal transduction pathway were displayed using GenMAPP 2 (<http://www.genmapp.org/>).

Stromal cell line proliferation and differentiation

Isolation and characterization of the uterine stromal cell lines have been described in detail previously (Piva *et al.* 1996). Cells (UIII) from the same passage (between passages 15 and 25) were used to determine treatment effects within an experiment by propagating a sufficient number of cells for each experiment in medium 199 containing 10% FBS (Piva *et al.* 1996). Quiescence was induced by culturing stromal cells for 72 h in serum-free, phenol red-free Dulbecco's modified eagle's medium (Gibco) and molecular cellular development biology (MCDB)-105 (Sigma-Aldrich) in a 3:1 mixture containing insulin (5 $\mu\text{g}/\text{ml}$) and supplements as detailed elsewhere (Piva *et al.* 1996). Quiescent cells were stimulated to synchronously enter the cell cycle by adding progesterone (1 μM), estradiol 17- β (10 nM), and fibroblast growth factor (FGF) (50 ng/ml). Proliferation was measured using the thiazolyl blue tetrazolium bromide (MTT) assay that has been validated previously in our laboratory (Piva *et al.* 1996). Quiescent stromal cells were stimulated to differentiate by adding progesterone (1 μM), estradiol 17- β

Table 1 Changes in effectors and targets of Wnt signal transduction in progesterone-pretreated uteri**Wnt pathway genes ratio**

Effectors	P/OVX	Targets	P/OVX
LDLR	1.2	Cyclin D1	1.07
JNK2	2.2	Cyclin D3	1.08
RhoA	1.0	uPA-R	1.3
PKC δ	1.12		
TCF-LEF	1.13		
Axin	1.24		
β -Trcp	1.7		
GSK-3 β	-1.7		
c-Jun	-1.3		
PKC ϵ	-1.59		

Uteri were excised from ovariectomized (OVX) and OVX rats ($n=3$ rats each) treated with progesterone (2 mg/daily) for 3 consecutive days. RNA was isolated and converted to biotin-labeled cDNA. The biotin-labeled cDNA was hybridized to rat high-density oligonucleotide arrays as detailed in the text. The ratio of normalized expression levels for progesterone/OVX (P/OVX) was calculated and the genes involved in Wnt signal transduction pathway were identified as detailed in the text. The negative sign indicates decreased expression levels compared with OVX uteri.

(10 nM), cholera toxin (10 ng/ml), and interleukin-11 (IL-11, 10 ng/ml) as detailed (Rider *et al.* 2005).

Statistical analysis

Differences among treatments for proliferation assays were examined by ANOVA and differences among means were determined through Scheffe's *post hoc* tests. The amount of LEF-1 immunoprecipitated with β -catenin was compared using the Mann-Whitney *U*-test. A *P* value of <0.05 was considered statistically significant.

Results*Progesterone influences effectors and targets of Wnt signal transduction*

Micoarray analysis was used to identify potential changes in the expression of Wnt signaling effectors and targets in the uteri of OVX and progesterone-pretreated rats (Table 1). The normalized expression levels for genes in the progesterone-pretreated endometrium were compared with genes expressed in the OVX uteri and calculated as a ratio

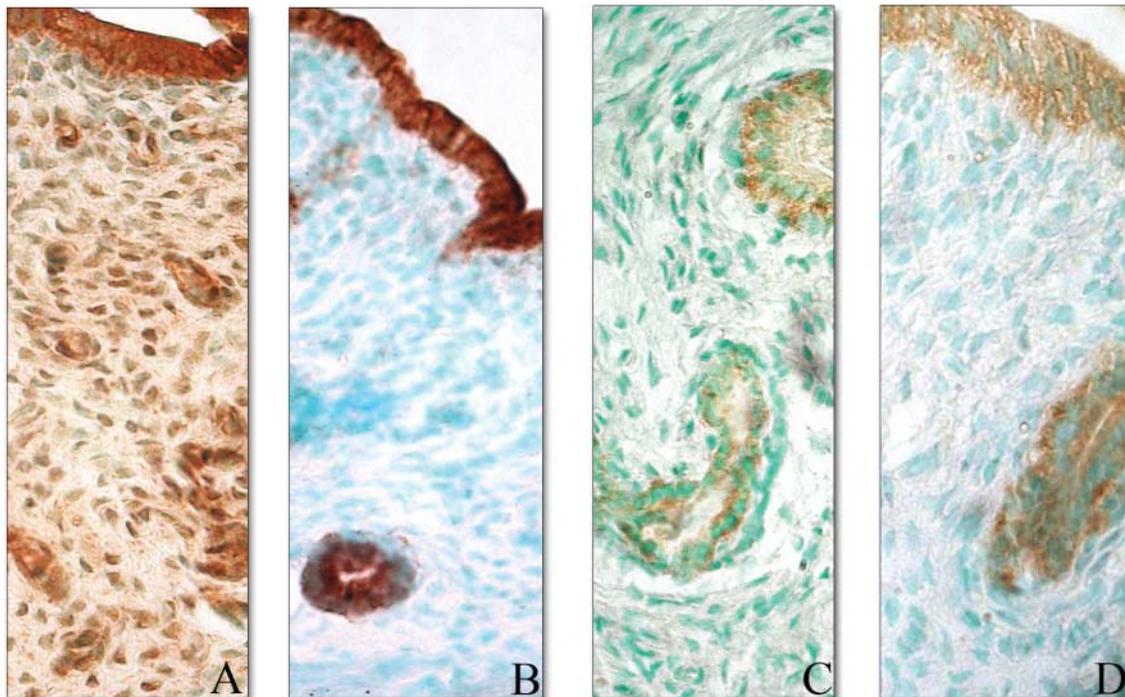


Figure 1 Progesterone down-regulates GSK-3 β expression in uterine stromal cells. The spatial distribution of total and phosphorylated (ortho) GSK-3 β was assessed in uterine sections from ovariectomized (OVX) and OVX rats treated with progesterone for 3 consecutive days (0 hE). Total GSK-3 β was evident in the stromal and the epithelial cells of OVX rats (A). There was strong immunoreactivity to ortho-GSK-3 β in the luminal and the glandular epithelium of OVX rat uteri but immunoreactivity in the stroma was absent (B). Total GSK-3 β expression was lost from the stroma and reduced in the epithelial cells at 72 h post-progesterone administration (C). Ortho-GSK-3 β was detected in the epithelial cells after progesterone pretreatment, but the stroma was negative (D). Original magnification, $\times 400$.

(progesterone/OVX). Progesterone increased the expression of seven Wnt effectors and decreased the expression of three effectors (indicated by the negative sign). There were 24 additional genes in the Wnt signal transduction pathway in which no detectable changes in expression were found (data not shown). Since the uterus is comprised of different cell types, gene profiling cannot identify which, if any, of these ratios are significant. However, we hypothesized that nuclear localization of cyclin D1 in uterine stromal cells required inactivation is GSK-3 β (Rider *et al.* 2003). Therefore, we investigated if down-regulation of GSK-3 β , as suggested by the microarray data, resulted in a cell-specific loss of GSK-3 β in progesterone-pretreated uteri.

Loss of GSK-3 β in the uterine stroma of progesterone-pretreated rats

The distribution of active and inactive GSK-3 β in the uteri of OVX rats and those treated for 3 consecutive days with progesterone (0 hE) were compared (Fig. 1). GSK-3 β was strongly expressed in the uterine stroma of OVX rats (Fig. 1A), while ortho-GSK-3 β (inactive) was not detected in the stromal cells (Fig. 1B). When rat uteri were treated with progesterone for 3 consecutive days, GSK-3 β expression was lost from the stroma. GSK-3 β intensity also declined in the epithelium although it remained detectable when compared with the greater loss of immunoreactivity in the

stroma (Fig. 1C). Inactive Ortho-GSK-3 β was not detected in the uterine stroma of progesterone-pretreated rat uteri but was evident in the luminal and the glandular epithelial cells (Fig. 1D). These results suggest that progesterone down-regulates GSK-3 β in the uterus but the loss is more evident in the stromal cells. Furthermore, Ortho-GSK-3 β is strongly expressed in the epithelial but not in the uterine stromal cells of OVX rat uteri.

GSK-3 β is expressed in uterine stromal cells during stromal cell entry into S and M phases of the cell cycle

A significant number of stromal cells in rat uteri pretreated with progesterone enter S phase of the cell cycle at 6 h post-estradiol administration and M phase at 12 h post-estradiol injection (Rider *et al.* 2003). Analysis of GSK-3 β distribution during these cell cycle phases revealed that GSK-3 β was expressed in the uterine stromal cells (Fig. 2A and B). The kinase was expressed primarily at the periluminal regions (Fig. 2A and B) where stromal cells are proliferating (Rider *et al.* 2003) and at the antimesometrial aspect of the uterus (not shown). Weaker immunoreactivity was evident throughout the stroma with faint GSK-3 β detected at the mesometrial region (not shown). Uterine epithelial cells expressed GSK-3 β at 6 and 12 h post-estradiol (Fig. 2A and B).

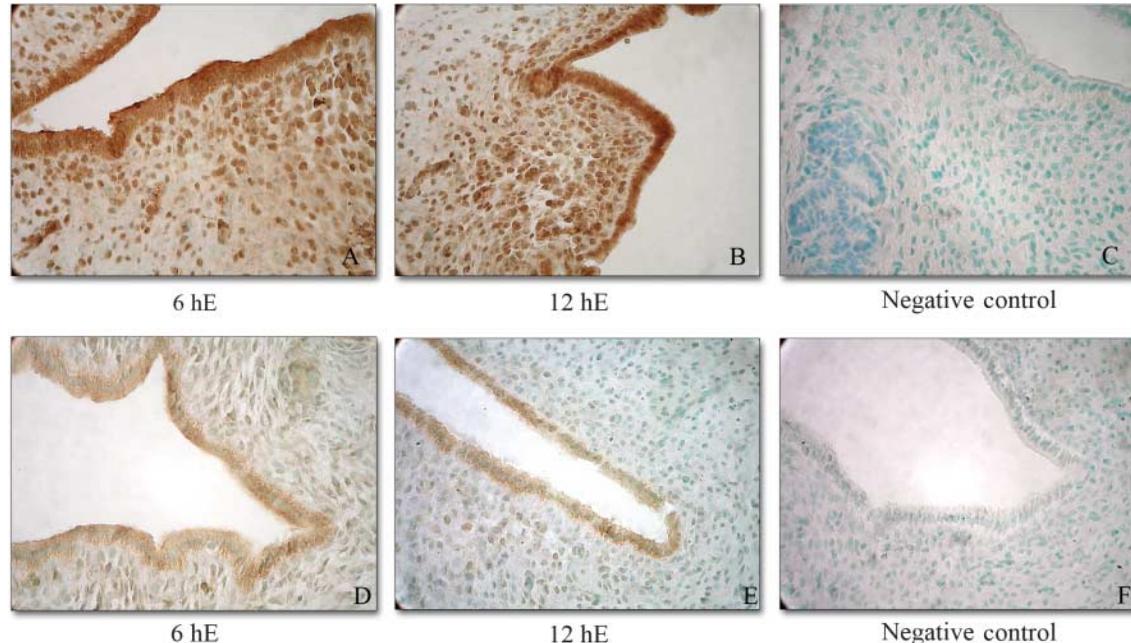


Figure 2 Active GSK-3 β is re-expressed in the uterine stroma of progesterone-pretreated rats after estradiol administration. Ovariectomized rats were treated with progesterone and estradiol as described in the text. The uterine horns were removed at 6 (A and D) and 12 (B and E) h post-estradiol administration and the distribution of total (A and B) and ortho (D and E) GSK-3 β was assessed using immunocytochemistry. Total GSK-3 β was strongly expressed in both the stromal and the epithelial cells, while ortho-GSK-3 β was weakly expressed in both the compartments. In the absence of primary antibody (C and F negative controls), total and ortho GSK-3 β immunoreactivity was absent. Original magnification, $\times 400$.

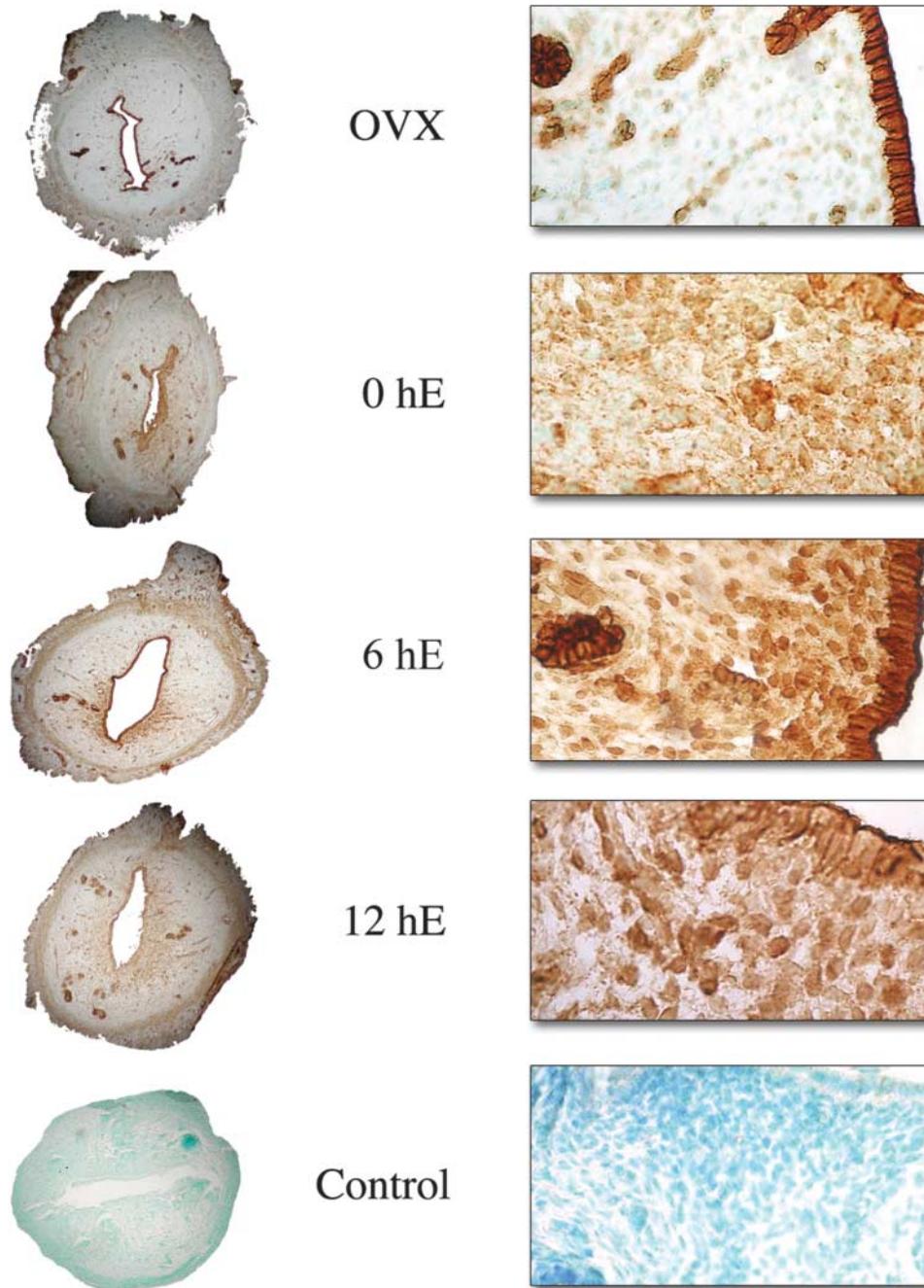


Figure 3 Progesterone pretreatment activates the canonical Wnt signal transduction pathway in uterine stromal cells. Ovariectomized (OVX) rats were treated with progesterone and estradiol as described in the text. The uterine horns were removed from OVX rats, those pretreated with progesterone for 72 h (0 hE), and those pretreated with progesterone followed by estradiol for 6 (6 hE) and 12 h (12 hE). β -Catenin was expressed in the epithelial cells at all time points and treatments. Stromal cells in the uteri of OVX rats did not contain β -catenin, but the protein was evident in the stroma after progesterone pretreatment (0 hE) particularly at the antimesometrial aspect (shown by higher magnification of this region). β -Catenin accumulation continued at 6 and 12 h post-estradiol in the antimesometrial and the periluminal stromal regions (shown by higher magnification of these regions). In the absence of primary antibody, there was no immunoreactivity (control). Original magnification left sections, $\times 200$; original magnification right sections, $\times 400$.

To determine if the GSK-3 β expressed in the stroma during S and M phases of the stromal cell cycle (Rider *et al.* 2003) was active, we utilized the ortho-GSK-3 β antibody. At both 6 and 12 h post-estradiol, ortho-GSK-3 β immunoreactivity was faint throughout the uterine stroma (Fig. 2D and E). Ortho-GSK-3 β immunoreactivity in the uterine epithelium was more strongly expressed when compared with that in the stroma (Fig. 2D and E).

Progesterone initiates Wnt- β -catenin signaling

Loss of GSK-3 β from the uterine stroma suggested that progesterone pretreatment stimulated the canonical Wnt- β -catenin signal transduction pathway. Since β -catenin appearance is the hallmark of the canonical Wnt signaling pathway, β -catenin expression was mapped in the uteri of OVX and hormone-treated rats (Fig. 3). β -Catenin was not evident in the stromal cells of OVX rats but after 3 days of progesterone injections, expression was evident in the uterine stroma, primarily in the antimesometrial and the periluminal regions (Fig. 3, 0 hE). The spatial distribution at these stromal regions continued in progesterone-pretreated uteri at 6 (6 hE) and 12

(12 hE) h post-estradiol injections. The protein was expressed in the glandular and the luminal epithelia of OVX and hormone-treated rat uteri at all times examined.

Estradiol stimulates nuclear localization of β -catenin

Although β -catenin accumulated in progesterone-pretreated uterine stromal cells, previous results from our laboratory showed that estradiol is required for stromal cells to enter the cell cycle (Rider *et al.* 2003). β -Catenin in progesterone-pretreated stromal cells was primarily found in the cytoplasm of the stromal cells (Fig. 4B). However, administration of estradiol stimulated β -catenin translocation to the nuclei of the antimesometrial and the periluminal stromal cells (Fig. 4C). Nuclear localization of β -catenin was a stromal cell-specific effect of estradiol because β -catenin in the luminal and the glandular epithelial cells remained in the cytosol (data not shown).

Estradiol stimulates β -catenin complex formation with TCF/LEF

β -Catenin forms a protein complex with TCF/LEF transcription factors on the promoters of Wnt-dependent

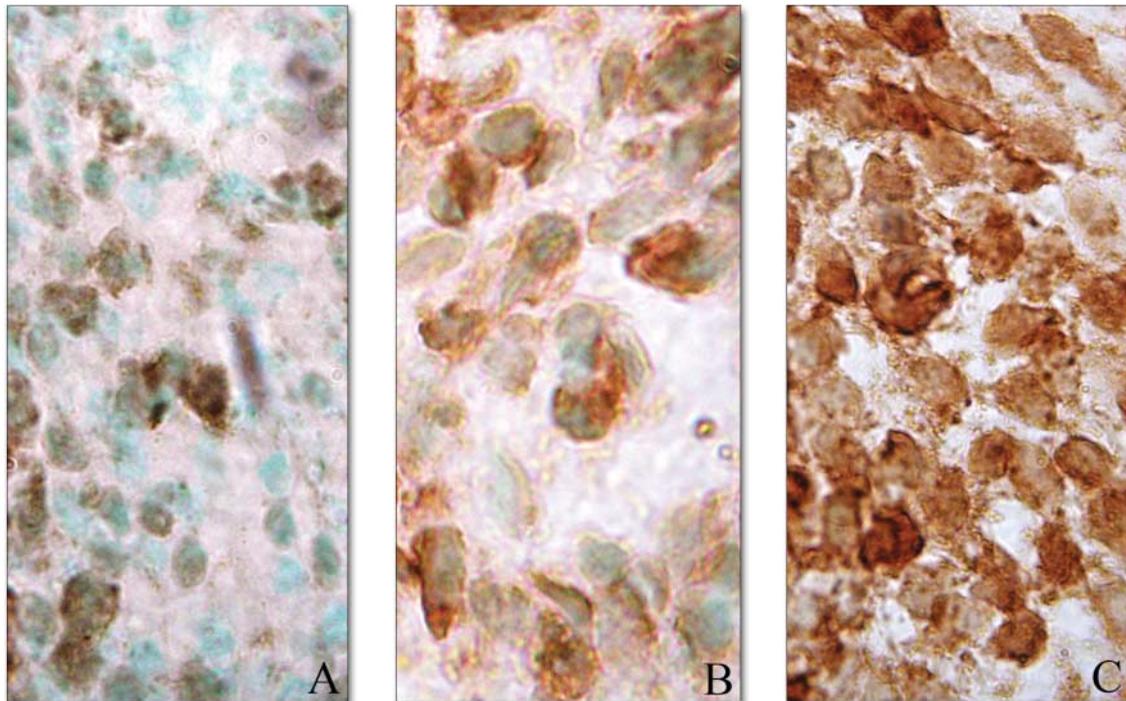


Figure 4 Nuclear localization of β -catenin is stimulated by estradiol administration. Ovariectomized (OVX) rats were treated with progesterone and estradiol as described in the text. The uterine horns were removed from OVX rats (A), those pretreated with progesterone for 72 h (B), and those pretreated with progesterone followed by estradiol for 6 h (C). The cell-specific distribution of β -catenin was assessed by immunocytochemistry. Progesterone pretreatment (B) stimulated the accumulation of β -catenin in the stromal cells located in the antimesometrial aspect of the endometrium. β -Catenin localized primarily in the cytoplasm as evidenced by the presence of methyl green-stained nuclei. Administration of estradiol to progesterone-pretreated rats (C) stimulated nuclear localization of β -catenin in the antimesometrial and the periluminal regions. Original magnification, $\times 1000$.

genes. To investigate if the estradiol-dependent nuclear localization of β -catenin resulted in complex formation with TCF/LEF, β -catenin was immunoprecipitated from uterine extracts from OVX rats, OVX rats pretreated with progesterone (0 hE) and with progesterone plus estradiol for 6 (6 hE) and 12 h (12 hE). The immunoprecipitates were size-fractionated, transferred to nitrocellulose membranes and the membranes were reacted with a LEF-1 antibody (Fig. 5A). A protein consistent with the size of mouse LEF-1 (58 kDa) was detected in extracts from OVX rats. This protein was not detected in the absence of LEF-1 antibody (data not shown). The amount of LEF-1 in the β -catenin immunoprecipitates increased in progesterone-pretreated uteri stimulated with estradiol for 6 and 12 h compared with the amount in progesterone-pretreated or OVX uteri. Re-precipitation of

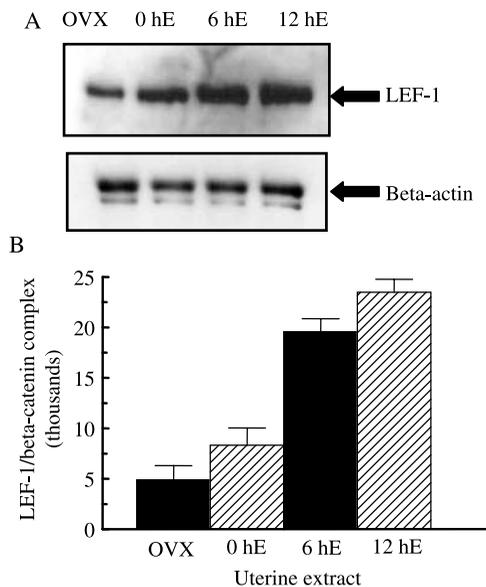


Figure 5 Estradiol stimulates β -catenin complex formation with lymphoid enhancer factor-1 (LEF-1). Rats were ovariectomized (OVX) and either pretreated with progesterone (2 mg daily) for 3 consecutive days ($n=5$) or pretreated with progesterone plus estradiol ($n=5$). The uterine horns were removed at 6 and 12 h post-estradiol administration. (A) β -Catenin was immunoprecipitated from uterine extracts. The immunoprecipitates were size-fractionated by SDS-PAGE and transferred to nitrocellulose by standard methods. The nitrocellulose membrane was reacted with a LEF-1 antibody and reactive proteins were visualized by chemiluminescence. Lane 1, extract from ovariectomized rat uteri (OVX); lane 2, extract from progesterone-pretreated uteri (0 hE); lane 3, extract from progesterone-pretreated uteri stimulated with estradiol for 6 h (6 hE); lane 4, extract from progesterone-pretreated uteri stimulated with estradiol for 12 h (12 hE). Arrow indicates LEF-1 protein that migrated with an apparent molecular mass of 58 kDa. To control for equal protein loading, β -actin (shown by arrow) was immunoprecipitated from the original extracts and analyzed on the same gel. (B) The amount of LEF-1 was measured by scanning densitometry of β -catenin immunoprecipitates in triplicate, reacted with LEF-1 antibody. Estradiol increased ($P<0.05$) the amount of LEF-1 bound to β -catenin compared with that in progesterone-pretreated or OVX rat uterine extracts.

uterine extracts with β -catenin was negative for detectable LEF-1 indicating the initial precipitation removed β -catenin-LEF complexes (data not shown). Quantitation of LEF-1 from triplicate samples revealed a significant increase ($P<0.05$) in β -catenin/LEF-1 in response to estradiol (Fig. 5B).

Sex steroids stimulate Wnt target gene expression

Microarray analysis suggested that progesterone stimulates the expression of three target genes in the progesterone-pretreated uterus (Table 1). We showed previously that cyclin D1 and D3 mRNA is expressed in the progesterone-pretreated rat endometrium but the amount was not significantly different from that measured in uteri after estradiol and entry into G1 phase of the stromal cell cycle (Rider *et al.* 2003). However, cyclin proteins were detected in stromal cell nuclei but only in response to estradiol and cell cycle entry. In order to investigate further the hormone-dependent activation of Wnt signaling that stimulates target gene expression, uPA-R was mapped in OVX, progesterone-pretreated and estradiol-injected rats (Fig. 6). In ovariectomized rat uteri, uPA-R was expressed in both the luminal and the glandular epithelium. Stromal cells, particularly those in the periluminal region, did not express uPA-R (Fig. 6A). The expression of uPA-R in the periluminal stromal cells increased in progesterone-pretreated uteri (Fig. 6B). At 12 h post-estradiol injection, uPA-R expression increased and extended into the deeper periluminal stromal cells (Fig. 6C).

β -Catenin expression increases in proliferating and differentiating uterine stromal cell lines in culture

Stromal cells in the rat uterus proliferate and differentiate into the decidua in response to sex steroids. Since most target genes involved in Wnt signaling are cell specific, it was of interest to investigate if progesterone-dependent activation of Wnt was restricted to those targets controlling cell proliferation. Rat uterine stromal cell lines proliferate in response to sex steroid plus growth factors (Piva *et al.* 1996) and differentiate in response to sex steroids and differentiation agents (Rider *et al.* 2005). To investigate if Wnt signaling was restricted to proliferating cells, the accumulation of β -catenin was compared between stromal cell simulated to proliferate and differentiate in culture to quiescent stromal cells (Fig. 7A). At 12 h post-stimulation, β -catenin accumulation increased in stromal cells stimulated to proliferate (lane 1) and differentiate (lane 2) compared with quiescent cells (lane 3). Scanning densitometry of samples in triplicate revealed a 1.5 (proliferating)- and 1.3 (differentiating)-fold increase of β -catenin in proliferating and differentiating cells compared with quiescent cells (data not shown). Analysis of the proliferative effects of these same agents showed that FGF plus sex steroids stimulated stromal cell proliferation ($P<0.05$), while stromal cells stimulated with sex steroids plus cholera toxin and IL-11 did not proliferate (Fig. 7B).

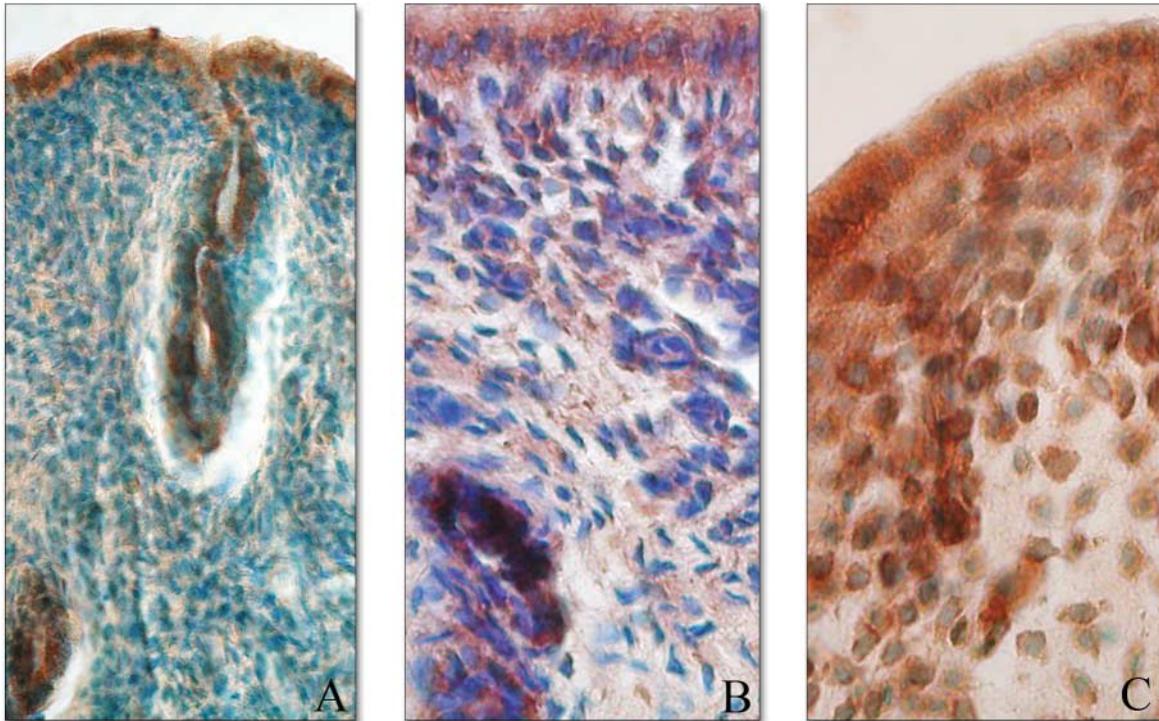


Figure 6 Urokinase plasminogen activator receptor (uPA-R) is a progesterone/Wnt target in uterine stromal cells. Ovariectomized (OVX) rats were treated with progesterone and estradiol as described in the text. The uterine horns were removed from OVX rats, those pretreated with progesterone for 72 h (0 hE), and those pretreated with progesterone and estradiol for 12 h (12 hE). The cell-specific distribution of uPA-R was assessed by immunocytochemistry. In OVX rat uteri, uPA-R was expressed in the luminal and the glandular epithelium (A). Expression uPA-R in the periluminal stromal cells was weak particularly in the periluminal region. Three days after progesterone pretreatment (B), uPA-R expression was evident in the periluminal stromal cells. Weaker expression of uPA-R extended deeper within the stroma (B). There was no apparent change of expression in the epithelial cells. At 12 h post-estradiol in progesterone-pretreated uteri, uPA-R expression was more evident than in progesterone-pretreated periluminal cells and extended deeper into the periluminal and the antimesometrial stromal cells (C).

Therefore, differentiation agents stimulated β -catenin accumulation in the absence of a proliferative response.

Discussion

Cell proliferation and differentiation in the mammalian uterus is regulated by the action of the female sex hormones estradiol and progesterone. The present results show that the administration of progesterone for 3 consecutive days to ovariectomized rats activates the canonical Wnt signal transduction pathway in uterine stromal cells, in part, by down-regulating the expression of GSK-3 β . Reduction in GSK-3 β , particularly in the antimesometrial and the periluminal stromal regions, leads to the accumulation of β -catenin in these cells. Stromal cells within these regions are destined to proliferate and differentiate into the decidua to form the maternal interface with the placenta. Previous studies from our laboratory showed that although progesterone prepares stromal cells to proliferate synchronously, progesterone-pretreated stromal cells do not enter the cell

cycle until estradiol administration (Rider *et al.* 2003). The present results suggest that the cell cycle entry requires nuclear localization of β -catenin and sufficient complex formation with LEF-1. These events are stimulated by estradiol.

Estradiol and progesterone regulate GSK-3 β expression and activity in a cell-specific manner in the uterus. GSK-3 β is strongly expressed in the luminal epithelial cells of OVX rats. However, much of the GSK-3 β is inactive based on the strong immunoreactivity to an Ortho-GSK-3 β antibody. Epithelial cells in OVX rats proliferate in response to estradiol (Martin & Finn 1968, Quarmby & Korach 1984) and estradiol is proposed to suppress GSK-3 β activity in mouse uterine epithelial cells (Chen *et al.* 2005a,b). Our data are consistent with the view that the activity of GSK-3 β is differentially controlled by sex steroids in the uterine epithelium. In the absence of sex steroids in the OVX rat uteri, epithelial GSK-3 β is phosphorylated. This inactivates the kinase and allows epithelial cells to proliferate in response to estradiol. When the endometrium is dominated by progesterone, GSK-3 β is down-regulated in both compartments. However, ortho

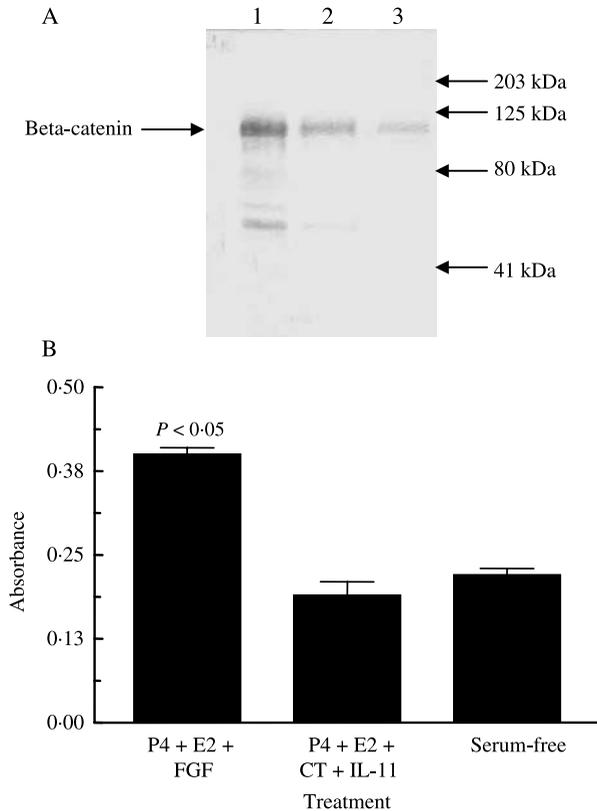


Figure 7 β -Catenin accumulates in proliferating and differentiating uterine stromal cells in culture. Quiescent uterine stromal cells (cell line U11) were stimulated to proliferate with progesterone (P4; 1 μ M), estradiol (E2; 10 nM), and basic FGF (50 ng/ml). Some cells were stimulated to differentiate using the same hormone treatment plus cholera toxin (CT; 10 ng/ml) and interleukin-11 (IL-11; 10 ng/ml). (A) Cells were collected 12 h after stimulation and extracts were immunoprecipitated with β -catenin. The immunoprecipitates were analyzed by western blotting. β -Catenin migrated with a molecular mass of approximately 102 kDa. Lane 1, stromal cells stimulated with proliferating agents; lane 2, stromal cells stimulated with differentiation agents; lane 3, quiescent stromal cells. Data shown are representative of samples analyzed in triplicate from three independent assays. (B) The proliferative response was measured 48 h after addition of test substances using the MTT assay. Data represent the mean \pm S.E.M. of three independent experiments in triplicate. $P < 0.05$ compared with quiescent cells.

GSK-3 β immunoreactivity also decreases in the epithelium. This suggests that in the progesterone-dominated endometrium, GSK-3 β is active in the luminal epithelial cells and could explain, in part, the inhibitory effects of progesterone on epithelial cell proliferation (Rider & Psychoyos 1994, Tong & Pollard 1999, Chen *et al.* 2005a).

Control of GSK-3 β activity in the uterus, particularly in the stroma, is unusual because progesterone down-regulates its expression. In most cell types, phosphorylation/dephosphorylation of GSK-3 β controls its activity. This mechanism does seem to be utilized in the epithelial cells but not in the stroma. Stromal cells within the endometrial regions that lack

GSK-3 β , accumulate β -catenin. This is compelling evidence that progesterone initiates Wnt signaling, in part, by down-regulating GSK-3 β . The reappearance of GSK-3 β at 6 and 12 h post-estradiol administration was unexpected. We anticipated that this GSK-3 β would be inactive but ortho-GSK-3 β was not detected in the uterine stromal cells at these times. We postulate that active GSK-3 β in the uterine stroma 6 and 12 h post-estradiol must help synchronize the proliferative response because only those stromal cells that are in G1 transit when GSK-3 β is re-expressed will continue through the cell cycle. In the presence of active GSK-3 β , no new cells will enter the cell cycle.

In order for β -catenin to act as a transcriptional co-activator, it must enter the nucleus and form a complex with sequence-specific DNA binding TCF/LEF transcriptional factors. In the absence of nuclear β -catenin, TCF/LEFs act as transcriptional repressors (Behrens *et al.* 1996, Brantjes *et al.* 2002, Mulholland *et al.* 2005). Our results show that estradiol stimulates β -catenin translocation to the nucleus and increases the amount of β -catenin bound to LEF-1. The simplest interpretation of this experiment is that cytoplasmic levels of β -catenin continue to accumulate in the presence of estradiol until a sufficient amount is reached and β -catenin translocates to the nucleus (Behrens *et al.* 1996, Widelitz 2004). However, it is possible that estradiol stimulates other signaling pathways or proteins that control the nuclear import of β -catenin. Insulin receptor substrate-1 (IRS-1) is a docking protein for insulin-like growth factor-1 (IGF-1) and insulin receptors. Activation of the IRS-signaling system stimulates mitosis and prevents apoptosis (reviewed in White 1998). IGF-1 stimulates the translocation of β -catenin to nuclei of R+ cells and IRS-1 is required for this nuclear import (Chen *et al.* 2005b). Of interest to the present study is the report by Morelli *et al.* (2004) indicating that the translocation of IRS-1 to nuclei is stimulated by estradiol in MCF-7 breast cancer cell line. It is now important to investigate further the mechanism (s) by which estradiol stimulates nuclear import of β -catenin.

Wnt proteins play critical roles in cell adhesion, cancer, and development (reviewed in Polakis 2000, Nusse 2005). The formation of highly specific regulatory complexes is essential for the cell-specific gene transcription necessary for differentiation. Wnt signaling controls the activation of many cell type-specific genes as well as those genes, such as cyclins D1, D3, and uPA-R that are induced in many cell types (Tetsu & McCormick 1999). We showed previously that cyclins D1 and D3 translocate to the nuclei of progesterone-pretreated stromal cells in response to estradiol (Rider *et al.* 2003) and that cyclin D1 mRNA increases significantly at 9, 12, and 15 h in uterine stromal cell lines in response to progesterone and FGF (Jones *et al.* 2000). Progesterone increases uPA-R mRNA (Table 1) and protein (Fig. 6) in the periluminal stromal cells. However, estradiol augments and extends the progesterone response consistent with the postulate that estradiol is required for full activation of Wnt signaling. It is worth noting that changes in expression in Wnt effectors and targets are modest. This is consistent with our data that show

changes in the expression of Wnt effectors and targets occur mainly in the stromal cells located in the periluminal and the antimesometrial stroma. Thus, small changes in gene expression, which are highly relevant for cell function, could be missed by gene profiling without complimentary localization studies. Microarray analysis as used in this study is also likely to underestimate the number of Wnt target genes. Signal transduction pathways that regulate stromal cell differentiation are largely unknown. Many Wnt target genes are cell specific and will only be identified by systematic comparisons between stromal and decidual cells. Decidual prolactin-related protein (dPRP) is a differentiation-specific marker for uterine stromal cells (Rider *et al.* 2005). Sex steroids and the differentiation agents' cholera toxin and IL-11 activate a dPRP reporter gene in these stromal cell lines (Rider *et al.* 2005). The present results show that β -catenin accumulates in differentiating stromal cells suggesting that the hormonal control of Wnt signaling activates target genes that regulate both proliferation and differentiation. The challenge is now to identify those targets that are required for differentiation of stromal cells.

In summary, this study reveals that progesterone activates Wnt signaling in uterine stromal cells. Activation of the canonical Wnt pathway results in the accumulation of β -catenin in the stromal cell cytoplasm. However, translocation of β -catenin and increased binding with LEF-1 occur in response to estradiol administration by mechanisms yet to be defined. In the uterine stroma, progesterone prepares the cells to enter the cell cycle by initiating Wnt signaling, but estradiol is required for full pathway activation and cell cycle entry.

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