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Journal of Cellular Biochemistry 107:1182–1188 (2009)

Journal of Cellular Biochemistry

Changes in Expression of Cell-Cycle-Related Genes in PC-3 Prostate Cancer Cells Caused by Ovine Uterine Serpin

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ABSTRACT

The hormonal-regulated serpin, ovine uterine serpin (OvUS), also called uterine milk protein (UTMP), inhibits proliferation of lymphocytes and prostate cancer (PC-3) cells by blocking cell-cycle progression. The present aim was to identify cell-cycle-related genes regulated by OvUS in PC-3 cells using the quantitative human cell-cycle RT^2 Profiler^{TM} PCR array. Cells were cultured ±200 μg/ml recombinant OvUS (rOvUS) for 12 and 24 h. At 12 h, rOvUS increased expression of three genes related to cell-cycle checkpoints and arrest (CDKN1A, CDKN2B, and CCNG2). Also, 14 genes were down-regulated including genes involved in progression through S (MCM3, MCM5, PCNA), M (CDC2, CKS2, CCNH, BIRC5, MAD2L1, MAD2L2), G_1 (CDK4, CUL1, CDKN3) and DNA damage checkpoint and repair genes RAD1 and RBPP8. At 24 h, rOvUS decreased expression of 16 genes related to regulation and progression through M (BIRC5, CCNB1, CKS2, CDK5RAP1, CDC20, E2F4, MAD2L2) and G_1 (CDK4, CDKN3, TFDP2), DNA damage checkpoints and repair (RAD17, BRCA1, BCCIP, KPNA2, RAD1). Also, rOvUS down-regulated the cell proliferation marker gene MKI67, which is absent in cells at G_0. Results showed that OvUS blocks cell-cycle progression through upregulation of cell-cycle checkpoint and arrest genes and down-regulation of genes involved in cell-cycle progression. J. Cell. Biochem. 107: 1182–1188, 2009.

KEY WORDS: UTERINE SERPIN; UTMP; SERPINA14; CELL-CYCLE ARREST; PC-3 PROSTATE CANCER CELLS; PCR-ARRAY; P21; PROLIFERATION

Uterine serpins (US), also known as uterine milk proteins (UTMP), are members of the serine proteinase inhibitor (serpin) superfamily [Ing and Roberts, 1989; Mathialagan and Hansen, 1996] and are designated as SERPINA14. These progesterone-induced glycoproteins are secreted in large quantities into the uterus of a restricted group of mammals during pregnancy [Leslie et al., 1990; Malathy et al., 1990; Tekin et al., 2005b]. The best studied of the US is the protein found in the sheep. Ovine uterine serpin (OvUS), which is the most abundant protein in uterine secretions of the pregnant sheep [Hansen et al., 1987a; Moffatt et al., 1987], is an example of a serpin that has gained a new function while apparently losing proteinase inhibitory activity characteristic of serpins. Other examples include the heat shock protein 47 [Nagata, 1998], corticosteroid and thyroxine binding globulin [Pemberton et al., 1998] and angiotensinogen [Morgan et al., 1996].

Inhibitory serpins inactivate their target proteinases by an irreversible suicide substrate-like mechanism after the proteinase binds to the reactive center loop (RCL) [Silverman et al., 2001]. Usually, inhibitory serpins are recognized by a consensus sequence in the hinge region which is localized within the RCL of the serpin [Irving et al., 2000] but the hinge region of OvUS is not conserved with inhibitory serpins [Irving et al., 2000; Tekin et al., 2005b]. Ovine US does not inhibit cathepsins B, D, and E [Mathialagan and Hansen, 1996], dipeptidyl proteinase IV [Liu and Hansen, 1995], trypsin, chymotrypsin, plasmin, thrombin, elastase, or plasminogen activator [Ing and Roberts, 1989]. While OvUS inhibits the aspartic proteinases pepsin A and C, this inhibition is atypical for serpins since an excess of 35- and 8-fold molar of OvUS was required for 50% inhibition of pepsin A and C, respectively [Mathialagan and Hansen, 1996].

The role of OvUS during pregnancy has been linked to the protection of the allogeneic conceptus against the maternal immune system [Hansen, 1998]. It exerts this role in large part by inhibiting the proliferation of activated-lymphocytes [Hansen et al., 1987b; Peltier et al., 1999]. The anti-proliferative effect of OvUS is also exerted on some other cell types including mouse lymphoma (P388D1), canine primary osteogenic sarcoma (D-17), and human prostate cancer (PC-3) cell lines [Tekin et al., 2005a, 2006].

The mechanism by which OvUS inhibits cell proliferation is poorly understood. Ovine US did not cause cytotoxic or apoptotic effects on lymphocytes or PC-3 cells [Skopets and Hansen, 1993; Tekin et al., 2005a; Padua and Hansen, 2008]. It was recently determined that OvUS blocks cell-cycle progression in mitogen-stimulated lymphocytes and increases the number of cells at

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Received 20 March 2009; Accepted 30 April 2009 • DOI 10.1002/jcb.22222 • © 2009 Wiley-Liss, Inc.
Published online 15 June 2009 in Wiley InterScience (www.interscience.wiley.com).

the G_0/G_1 stage at 96 h after addition of protein [Padua and Hansen, 2008]. Ovine US also blocks the progression of the cell cycle of PC-3 cells in a manner that leads to accumulation of cells at G_2/M at 12 h after addition of the protein and at G_0/G_1 at 24 h after treatment [Padua and Hansen, 2008]. The objective of the present study was to understand the mechanism by which OvUS blocks cell-cycle progression by determining effects of OvUS on cell-cycle-related gene expression in PC-3 cells.

MATERIALS AND METHODS

MATERIALS

The FreeStyle™ 293 expression medium, Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 (DMEM-F12) and 0.25% Trypsin–EDTA were purchased from Gibco-Invitrogen (Carlsbad, CA). The G418 disulfate (geneticin) was purchased from Research Products International (Mount Prospect, IL), nickel Sepharose chromatography medium (high performance) from Amersham Biosciences (Piscataway, NJ), Precast Tris–HCl gradient Ready gels® were obtained from BioRad (Richmond, CA) and Centricon® filter devices were from Millipore Corporation (Bedford, TX). The human prostate cancer (PC-3) cell line was from ATCC (Rockville, MD), [^{3}H]thymidine (6.7 Ci/mmol) was from ICN (Irvine, CA) and fetal bovine and horse sera from Atlanta Biologicals (Norcross, GA). Other reagents were obtained from either Fisher (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO).

PURIFICATION OF RECOMBINANT OvUS

Human embryonic kidney (HEK)-293F (Gibco-Invitrogen) cells transfected with a plasmid construct containing the gene for OvUS [Tekin et al., 2006] were cultured continuously in selective medium (FreeStyle™ 293 expression medium containing 700 μg/ml of geneticin) at 37°C in a humidified incubator containing a gas environment of 8% (v/v) CO_2 in air. The recombinant (r)OvUS was purified by using immobilized metal ion (nickel) exchange chromatography as described by Padua and Hansen [2008]. Briefly, rOvUS was eluted with 20 mM phosphate buffer, 500 mM imidazole, 0.3 M NaCl, pH 8.0, concentrated and buffer-exchanged into Dulbecco's phosphate-buffered saline (DPBS) using Centricon plus-20® concentration devices. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions using 4–15% polyacrylamide Tris–HCl gradient gels and Coomassie Blue were used to assess the purity of the rOvUS. After filter-sterilization of rOvUS using 0.22 μm micro centrifuge devices, the concentration of the protein was determined by Bradford assay [Bradford, 1976] using bovine serum albumin as standard.

PC-3 CELL CULTURE

The PC-3 cell line was cultured in 75 cm^2 flasks continuously in complete medium [DMEM-F12 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 200 U/ml penicillin and 2 mg/ml streptomycin] at 37°C in a humidified incubator with a gas environment consisting of 5% (v/v) CO_2 in air. Cells were trypsinized after reaching 50–70% of confluence, centrifuged at 110*g* for 5 min and resuspended in fresh medium. Cell viability was assessed by trypan blue exclusion and cell concentration was adjusted according to each experiment.

PROLIFERATION ASSAY

PC-3 cells were cultured in 96-well plates in a volume of 100 μl and at a final concentration of 1×10^4 cells/ml. After 24 h in culture, cells were treated with either rOvUS, ovalbumin (OVA, a control serpin dissolved in DPBS) or vehicle (DPBS). The vehicle was added to control wells at an equivalent volume (6–20 μl) as for the rOvUS and OVA. Additional culture medium was added to all wells to bring the final volume to 200 μl. The final concentration of rOvUS and OVA was 200 μg/ml. After 48 h of culture, an aliquot of 10 μl of culture medium containing 0.1 μCi [^{3}H]thymidine was added to the wells. Cells were collected on fiber glass filters using a cell harvester (Brandel, Gaithersburg, MD) 24 h after [^{3}H]thymidine addition. Radioactivity on the filters was counted by scintillation spectrometry (Beckman Coulter, Inc., Fullerton, CA). The experiment was replicated on five different occasions using a total of four different batches of rOvUS. For each replicate, each treatment was tested in triplicate.

CELL CULTURE FOR RNA EXTRACTION

PC-3 cells were cultured in 4-well plates at a final concentration of 4×10^5 cells/ml in 100 μl. After 24 h of culture, treatments and complete medium were added to achieve a final concentration of 200 μg/ml rOvUS or an equivalent volume of DPBS as experimental control in a final volume of 400 μl. At 12 or 24 h after treatment addition, medium was removed from the plates and cells were lysed for total RNA cell extraction as described below. The experiment was replicated four times, with a different batch of rOvUS for each replicate.

RNA EXTRACTION

Total RNA was extracted using the RNeasy® plus micro kit (Qiagen, Inc., Valencia, CA) following the manufacturer's instructions. Briefly, PC-3 cells were lysed in wells for 5 min by repeat pipetting using 350 μl of lysis buffer supplied in the kit. Cell lysates were transferred into microcentrifuge tubes, vortexed for 1 min and placed into gDNA eliminator columns to remove genomic DNA. After mixing with 70% (v/v) ethanol, samples were transferred onto RNeasy MinElute spin columns, washed and total RNA eluted with RNase-free water. RNA concentration and quality was determined using an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) at the Gene Expression Core Laboratory of the Interdisciplinary Center of Biotechnology Research, University of Florida. High-quality RNA was used for RT-PCR array experiments (RNA integrity numbers $\geq$8.0).

cDNA SYNTHESIS AND REAL TIME-PCR ARRAY

The cDNA for each RNA sample was obtained using the Super Array RT^2 First Strand kit (SABiosciences Corporation, Frederick, MD) according to the manufacturer's instructions. Briefly, after genomic DNA elimination, the reverse transcription reaction was performed at 42°C for 15 min and then heated at 95°C for 5 min to inactivate the enzyme. The cDNA was mixed with RT^2 SYBR green/ROX qPCR master mix (SABiosciences Corporation) and 25 μl

TABLE I. Cell-Cycle-Related Genes Screened using the RT^2 Profiler^{TM} PCR Array

ABL1	ANAPC2	ANAPC4	DIRAS3	ATM	ATR	BAX	BCCIP	BCL2	BIRC5	BRCA1	BRCA2
CCNB1	CCNB2	CCNC	CCND1	CCND2	CCNE1	CCNF	CCNG1	CCNG2	CCNH	CCNT1	CCNT2
CDC16	CDC2	CDC20	CDC34	CDK2	CDK4	CDK5R1	CDK5RAP1	CDK6	CDK7	CDK8	CDKN1A
CDKN1B	CDKN2A	CDKN2B	CDKN3	CHEK1	CHEK2	CKS1B	CKS2	CUL1	CUL2	CUL3	DDX11
DNM2	E2F4	GADD45A	GTF2H1	GTSE1	HERC5	HUS1	KNTC1	KPNA2	MAD2L1	MAD2L2	MCM2
MCM3	MCM4	MCM5	MKI67	MNAT1	MRE11A	NBN	PCNA	RAD1	RAD17	RAD51	RAD9A
RB1	RBBP8	RBL1	RBL2	RPA3	SERTAD1	SKP2	SUMO1	TFDP1	TFDP2	TP53	UBA1
B2M[a]	HPRT1[a]	RPL13A[a]	GAPDH[a]	ACTB[a]	HGDC[b]	RTC[c]	RTC[c]	RTC[c]	PPC[d]	PPC[d]	PPC[d]

[a]House keeping genes.
[b]Human genomic DNA contamination control.
[c]Reverse transcription control.
[d]Positive PCR control.

aliquots were loaded into each well of the RT^2 Profiler PCR Array (SABiosciences Corporation, catalog number PAHS-020A). The PCR array was designed to study the profile of 84 human cell-cycle-related genes (Table I). PCR array experiments were performed on an ABI 7300 instrument (Applied Biosystems, Foster City, CA). Conditions for amplification were as follows: 1 cycle of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

STATISTICAL ANALYSIS

The General Linear Models procedure of SAS (SAS for Windows, Version 9.0; SAS Institute, Cary, NC) was used to analyze the data from the proliferation experiments by the least-square means analysis of variance. All main effects were considered fixed and the model included effects of treatments and batch of rOvUS. Differences between levels of a treatment were determined by the *P*diff mean separation test of SAS.

The PCR array data were analyzed by the ΔΔCt method. Genes with Ct values greater than 35 cycles were considered as non-detectable and assigned a value of 35. Average of four house keeping genes [beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase 1 (HPRT1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin (ACTB)] was used to obtain the ΔCt value for each gene of interest. The ΔΔCt value for each gene was calculated by the difference between the ΔCt of the treated and the ΔCt of the control groups. The fold-change for each gene was calculated by $2^{-\Delta\Delta Ct}$ and the statistical analysis to determine differences between treatments was performed using the RT^2 Profiler PCR Array Data Analysis web-based software (SABiosciences Corporation).

RESULTS

INHIBITION OF PC-3 CELL PROLIFERATION BY rOvUS

The inhibitory effect of rOvUS on the proliferation of PC-3 cells is shown in Figure 1. Incorporation of [^{3}H]thymidine into DNA was reduced ($P < 0.05$) by rOvUS. In contrast, the control serpin OVA did not affect [^{3}H]thymidine incorporation.

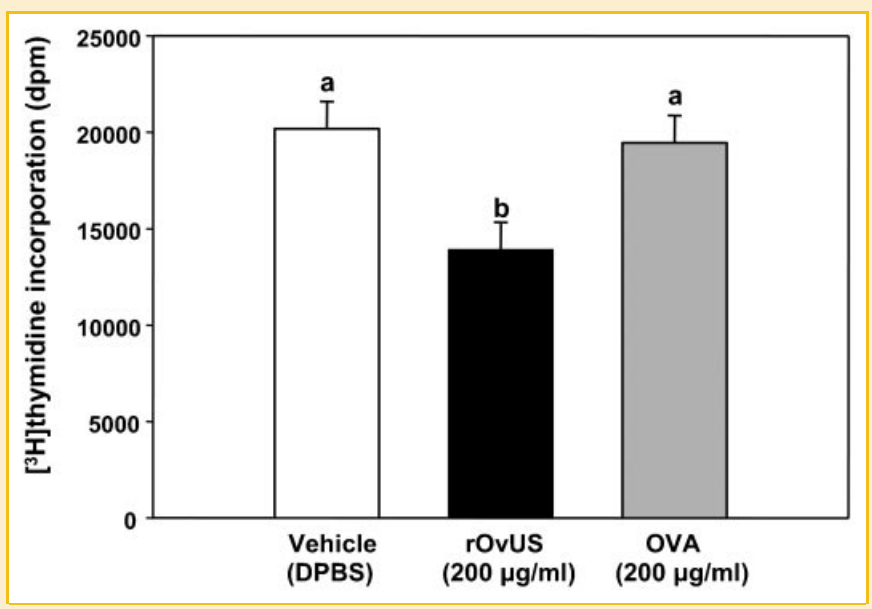

Fig. 1. Inhibition of [^{3}H]thymidine incorporation of PC-3 cells by 200 µg/ml of the recombinant ovine uterine serpin (rOvUS). Ovalbumin (OVA) was used as control serpin and Dulbecco's phosphate-buffered saline (DPBS) as vehicle. Data represent least-squares means ± SEM. Bars with different letters differ ($P < 0.05$).

TABLE II. Regulation of Cell-Cycle-Related Genes of PC-3 Cells after 12 h of Treatment with 200 μg/ml Recombinant Ovine Uterine Serpin

Gene symbol	Description	AVGΔCt ± SE Control	AVGΔCt ± SE Treatment	Fold change	95% CI	*P*-value
Up-regulated						
CCNG2	Cyclin G2	7.545 ± 0.29	6.134 ± 0.31	2.6585	(1.12, 4.20)	<0.05
CDKN1A	CDK inhibitor 1A (p21, Cip1)	4.078 ± 0.05	2.727 ± 0.24	2.5502	(1.70, 3.40)	<0.01
CDKN2B	CDK inhibitor 2B, p15	8.25 ± 0.36	7.037 ± 0.32	2.3184	(0.81, 3.83)	<0.05
Down-regulated						
BIRC5	Survivin	6.885 ± 0.11	7.514 ± 0.16	0.6465	(0.47, 0.82)	<0.05
CCNH	Cyclin H	3.783 ± 0.03	4.042 ± 0.06	0.8354	(0.76, 0.91)	<0.01
CDC2	Cell division cycle 2	1.45 ± 0.07	1.844 ± 0.06	0.7608	(0.67, 0.85)	<0.01
CDK4	Cyclin-dependent kinase 4	3.45 ± 0.06	3.942 ± 0.08	0.7111	(0.61, 0.81)	<0.01
CDKN3	Cyclin-dependent kinase inhibitor 3[a]	1.265 ± 0.05	1.519 ± 0.07	0.8384	(0.74, 0.94)	<0.05
CKS2	CDC28 protein kinase regulatory subunit 2	1.13 ± 0.07	1.437 ± 0.06	0.8084	(0.71, 0.91)	<0.05
CUL1	Cullin 1	4.375 ± 0.07	4.629 ± 0.06	0.8384	(0.74, 0.94)	<0.05
MAD2L1	MAD mitotic deficient-like 1	3.06 ± 0.06	3.622 ± 0.17	0.6774	(0.51, 0.85)	<0.05
MAD2L2	MAD mitotic deficient-like 2	4.593 ± 0.06	4.944 ± 0.09	0.7836	(0.66, 0.90)	<0.05
MCM3	Minichromosome maintenance deficient 3[b]	2.563 ± 0.17	3.632 ± 0.25	0.4765	(0.28, 0.67)	<0.05
MCM5	Minichromosome maintenance deficient 5[c]	5.965 ± 0.16	6.604 ± 0.05	0.642	(0.50, 0.79)	<0.05
PCNA	Proliferating cell nuclear antigen	2.225 ± 0.20	2.909 ± 0.08	0.6223	(0.44, 0.80)	<0.05
RAD1	RAD1 homolog (*S. pombe*)	7.41 ± 0.06	7.719 ± 0.07	0.807	(0.71, 0.91)	<0.05
RBBP8	Retinoblastoma binding protein 8	4.233 ± 0.15	4.927 ± 0.15	0.618	(0.44, 0.80)	<0.05

[a]CDK2-associated dual specificity phosphatase.
[b]Mitotin (*S. cerevisiae*).
[c]Cell division cycle 46 (*S. cerevisiae*).

CELL-CYCLE-RELATED GENE EXPRESSION PROFILE AT 12 H AFTER TREATMENT WITH rOvUS

The mRNA expression of 17 genes was altered ($P < 0.05$ or $P < 0.01$) by rOvUS (Table II). Expression of three genes involved in cell-cycle checkpoint and arrest were upregulated. These genes were CDKN1A ($p21^{cip1}$), CCNG2 (cyclin G2), and CDKN2B ($p15^{ink}$). In addition, the mRNA for 14 genes was decreased by rOvUS. Included in this group were three genes (MCM3, MCM5, and PCNA), the product of which are required at the S phase of the cell cycle for DNA synthesis and replication. Others are genes involved in the regulation and progression at the M phase [CDC2, CKS2, CCNH (cyclin H), BIRC5 (survivin), MAD2L1, MAD2L2] and at the G_1 phase (CDK4, CUL1, CDKN3). The last two genes of the 14 for which expression was down-regulated were DNA damage checkpoint and repair genes RBBP8 and RAD1.

CELL-CYCLE-RELATED GENE EXPRESSION PROFILE AT 24 H AFTER TREATMENT WITH rOvUS

Treatment of PC-3 cells with rOvUS for 24 h down-regulated ($P < 0.05$ or less) expression of 16 genes (Table III). Some of them (BIRC5, CDK4, CDKN3, CKS2, MAD2L2, and RAD1) were also down-regulated at 12 h. The others are genes related to the regulation and progression through the M (CCNB1, CDK5RAP1, CDC20, and E2F4) and G_1 (CDK4 and TFDP2). Likewise, rOvUS down-regulated expression of three DNA damage checkpoint related genes (RAD17, KPNA2, and BRCA1), activation of which blocks cell-

TABLE III. Down-Regulation of Human Cell-Cycle-Related Genes of PC-3 Cells after 24 h of Treatment with 200 μg/ml Recombinant Ovine Uterine Serpin

Gene symbol	Description	AVGΔCt ± SE Control	AVGΔCt ± SE Treatment	Fold change	95% CI	*P*-value
Down-regulated						
BCCIP	BRCA and CDKN1A interacting protein	2.63 ± 0.16	3.17 ± 0.10	0.6881	(0.51, 0.87)	<0.05
BIRC5	Survivin	6.21 ± 0.09	7.32 ± 0.29	0.4643	(0.27, 0.65)	<0.05
BRCA1	Breast cancer 1, early onset	6.41 ± 0.16	7.29 ± 0.30	0.5445	(0.29, 0.80)	<0.05
CCNB1	Cyclin B1	1.10 ± 0.06	2.00 ± 0.30	0.537	(0.31, 0.76)	<0.01
CDC20	Cell division cycle 20 homolog	0.53 ± 0.20	1.55 ± 0.17	0.4916	(0.32, 0.67)	<0.01
CDK4	Cyclin-dependent kinase 4	2.88 ± 0.11	3.41 ± 0.12	0.6941	(0.54, 0.85)	<0.05
CDK5RAP1	CDK5 regulatory subunit associated protein 1	9.07 ± 0.19	9.65 ± 0.13	0.6704	(0.46, 0.88)	<0.05
CDKN3	Cyclin-dependent kinase inhibitor 3[a]	1.02 ± 0.01	1.68 ± 0.24	0.6354	(0.43, 0.84)	<0.05
CKS2	CDC28 protein kinase regulatory subunit 2	0.91 ± 0.08	1.64 ± 0.20	0.6042	(0.43, 0.78)	<0.05
E2F4	ETF transcription factor 4	7.14 ± 0.14	7.62 ± 0.07	0.7148	(0.57 0.86)	<0.05
KPNA2	Karyopherin α 2 (RAG cohort 1, importin α1)	1.97 ± 0.23	2.89 ± 0.25	0.5269	(0.29, 0.77)	<0.05
MAD2L2	MAD2 mitotic arrest deficient-like 2	3.99 ± 0.08	4.69 ± 0.04	0.6148	(0.54, 0.69)	<0.001
MKI67	Antigen identified by monoclonal antibody Ki-67	3.35 ± 0.24	4.48 ± 0.34	0.4579	(0.20, 0.72)	<0.05
RAD1	RAD1 homolog (*S. pombe*)	6.96 ± 0.06	7.33 ± 0.13	0.7741	(0.62, 0.92)	<0.05
RAD17	RAD17 homolog (*S. pombe*)	6.38 ± 0.06	6.56 ± 0.02	0.8831	(0.80, 0.96)	<0.05
TFDP2	Transcription factor Dp-2	7.21 ± 0.09	7.49 ± 0.04	0.8211	(0.71, 0.93)	<0.05

[a]CDK2-associated dual specific phosphatase.

cycle progression at all stages of the cell cycle. Expression of BCCIP (BRCA2 and CDKN1A interacting protein) a gene involved in DNA repair, spindle formation and cytokinesis, was also down-regulated by rOvUS. In addition, rOvUS down-regulated MKI67 expression. The gene product of MKI67 (Ki-67) is a marker of cell proliferation.

DISCUSSION

It was previously shown that OvUS inhibited cell proliferation of PC-3 cells by disrupting cell-cycle progression [Padua and Hansen, 2008]. Results presented in this study corroborate those findings and provide an overview of the changes in gene expression that are associated with alterations in the cell cycle. In particular, inhibition of cell-cycle progression is initially associated with increased expression of genes that block cell cycle and decreased expression of genes needed for progression through G_1, S, and M phases. After more prolonged treatment, inhibition of gene expression required for cell-cycle progression is extended to a wider range of genes.

In an earlier study, OvUS caused accumulation of PC-3 cells at the G_2/M phase at 12 h after treatment [Padua and Hansen, 2008]. It is clear from examination of Figure 2A that different phases of the cell cycle are disrupted by OvUS at 12 h. Ovine US increased expression of CDKN1A ($p21^{cip1}$), CDKN2B ($p15^{ink}$) and CCNG2 (cyclin G2), all of which are involved in cell-cycle checkpoint and arrest. It is likely that upregulated expression of these genes is the proximal cause for the inhibition of cell-cycle progression. For example, CDKN1A belongs to the cyclin dependent kinase inhibitor (CDKI) family and inhibits cell-cycle progression by inhibiting CDK2 and CDK4 and by blocking DNA replication and repair by binding to PCNA [Harper et al., 1993; Li et al., 1994; Waga et al., 1994]. This inhibitor of cell-cycle progression causes arrest at G_1, S, and G_2 phases [Harper et al., 1993; Cayrol et al., 1998]. CDKN2B also belongs to the CDKI family and binds to CDK4 and CDK6 to prevent their association with cyclin D, thereby blocking the cell cycle at G_1 [Krug et al., 2002]. Finally, CCNG2 (cyclin G2) a non-typical cyclin for which expression is independent of p53 [Bates et al., 1996] blocks cell-cycle progression at the G_1/S phase by association with the active protein phosphatase 2A [Bennin et al., 2002]. CCNG2 gene is also expressed at the late S and G_2 phases [Le et al., 2007].

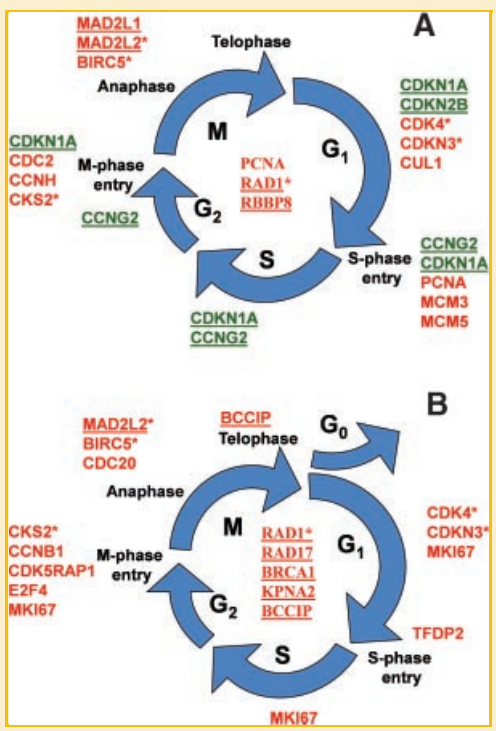

Fig. 2. Diagram illustrating points in the cell cycle where genes were differentially regulated by ovine uterine serpin at 12 (A) and 24 (B) h. Up-regulated genes are in green and down-regulated genes in red. Genes that block cell-cycle progression are underlined. Genes that were regulated at 12 and 24 h are shown with an asterisk (*). Genes involved in DNA repair are in the center of the cycle to represent that many of these are involved in several stages of the cell cycle.

At 12 h, OvUS down-regulated expression of genes involved in DNA replication (PCNA, MCM3, and MCM5) at the S phase as well as genes implicated in regulation and progression of the cell cycle at M (CDC2, CCNH, MAD2L1, MAD2L2) and G_1 (CDK4, CUL1, CDKN3) phases (Fig. 2A). As an example, CCNH (cyclin H) is the regulatory subunit of the cdk-activating kinase (CAK) and is distinct from mitotic cyclins because it is expressed constantly throughout the cell cycle. The function of cyclin H is related to the phosphorylation of different cyclin-dependent kinases (CDKs) and components of the transcriptional machinery [Kaldis, 1999].

At 24 h, down-regulation of cell-cycle-related genes became more widespread (Fig. 2B). Some genes for which expression was decreased by OvUS at 12 h remained down-regulated at 24 h (BIRC5, CDK4, CDKN3, CKS2, MAD2L2, and RAD1). However, reduced expression of other genes at 24 h post-treatment, including CCNB1, CDK5RAP1, CDC20, E2F4, TFDP2, RAD17, KPNA2, BRCA1, BCCIP and MKI67, was also observed. Down-regulation of expression of genes such as CCNB1, BIRC5, CDK4, CDC20, and TFDP2 would impede progression through the G_1, S, and G_2/M phases (Fig. 2B). For example, CDC20 is the activator of the anaphase-promoting complex/cyclosome (APC/C) required for the metaphase–anaphase progression during mitosis [Baker et al., 2007]. Expression of CDC20 was reduced by 50% by OvUS.

The down-regulation of MKI67 at 24 h suggests that a proportion of PC-3 cells entered the resting state (G_0) since the gene product for MKI67 (Ki-67) is a cell marker linked to proliferation and is present in all stages of the cell cycle with the exception of the G_0 stage [Gerdes et al., 1984]. This is consistent with the global decrease in gene expression observed at 24 h and also with earlier observations where OvUS caused an increase in the proportion of PC-3 cells at G_0/G_1 phase at 24 h after treatment [Padua and Hansen, 2008].

Upregulation of expression of CDKN1A ($p21^{cip}$), CDKN2B ($p15^{ink}$), and CCNG2 (cyclin G2) at 12 h is likely to be a cause for the down-regulated expression of genes at 12 and 24 h. It has been shown that high levels of CDKN1A ($p21^{cip}$) expression down-regulates expression of BIRC5 (survivin) in other cells [Lühr et al., 2003; Xiong et al., 2008]. It is also possible that down-regulation of expression of some genes inhibited the transcription of others. As an example, the down-regulation of CKS2 caused a reduction in the transcription of

CCNB1 (cyclin B1) and CDC2 (CDK1) [Martinsson-Ahlzén et al., 2008]. The activity of CDK1 is required for the regulation of some DNA repair pathways [Aylon et al., 2004; Branzei and Foiani, 2008].

The lack of functional p53 on PC-3 cells [Isaacs et al., 1991] probably affects feedback loops in response to OvUS. This protein is an effector molecule in the DNA repair pathway and lack of p53 abolishes the DNA checkpoints response and apoptosis [Sancar et al., 2004; Gatz and Wiesmüller, 2006]. Moreover, cells with disrupted p53 can overcome controls at the G_2/M checkpoint and fail to maintain a sustained arrest at this stage [Bunz et al., 1998].

All genes studied that are related to DNA damage checkpoints or repair (RBBP8, RAD1, RAD17, BRCA1, KPNA2, PCNA, and BCCIP) were down-regulated at either 12 or 24 h after OvUS treatment. The products of these genes are induced in response to incomplete DNA replication or damage at either specific or several points of the cell cycle [Li et al., 1994; Teng et al., 2003; Sancar et al., 2004; Lu et al., 2005; Sartori et al., 2007]. Thus, OvUS disrupts the transcription of some genes involved in nucleotide excision, mismatch, homologous recombination repair and translesion synthesis pathways in addition to the sensor (RAD1 and 17) and mediator molecules (BRCA1) of the DNA damage checkpoint. Failure of OvUS to induce apoptosis in PC-3 cells [Tekin et al., 2005a; Padua and Hansen, 2008] despite these changes in gene expression could reflect the lack of p53 or an as yet undescribed anti-apoptotic action of OvUS.

Ovine US is one of the few serpins identified that alters cell-cycle dynamics. Other is MENT, an intracellular protein with a very basic isoelectric point (9 vs. 5–6.5 for other serpins) [Silverman et al., 2001]. MENT inhibits the enzymatic activity of the nuclear cysteine proteinase SPase, a cathepsin L-like proteinase involved in degradation of the phosphorylated form of the retinoblastoma (Rb) protein, a regulator of the cell cycle [Irving et al., 2002]. Another intracellular serpin, PAI-2, can protect Rb from degradation through an independent anti-proteinase mechanism [Croucher et al., 2008]. Unlike MENT and PAI-2, OvUS is an extracellular protein that can bind to cell membranes [Liu et al., 1999]. Nonetheless, it is possible that OvUS inhibits cell proliferation by being internalized. Other extracellular serpins can become internalized and affect cell function. An example is α1-antitrypsin, which enters and resides in the cytoplasm of a mouse insulinoma cell line and protects against apoptosis through inhibition of caspase-3 activation [Zhang et al., 2007].

Alternatively, OvUS may block cell proliferation through inhibition or induction of signal transduction systems. In lymphocytes, OvUS inhibits proliferation of phorbol myristol acetate stimulated lymphocytes, suggesting that the protein blocks downstream actions of the protein kinase (PK) C pathway [Peltier et al., 1999]. Like OvUS, transforming growth factor (TGF)-β causes cell-cycle arrest by upregulation of CDKN2B ($p15^{ink}$), CDKN1A ($p21^{cip1}$), and CCNG2 (cyclin G2) [Horne et al., 1997; Gartel and Tyner, 2002]. Interferon (IFN)-γ also induces CDKN1A ($p21^{cip1}$) expression, independent of p53 by the STAT-1 pathway [Horne et al., 1997]. Both, TGF-β and IFN-γ signaling can cause transactivation of p21 by a p53-independent mechanism, where p21 protects cells against p53-independent apoptosis which is induced by these signals [Horne et al., 1997]. Signal transduction pathways affected by OvUS have not been determined. However, OvUS may regulate components of signaling pathways shared with TGF-β or IFN-γ pathways.

In summary, results presented here support the idea that OvUS inhibits proliferation of PC-3 cells by disrupting cell-cycle dynamics. Disruption involves increased expression of cell-cycle checkpoint and arrest genes CDKN1A ($p21^{cip1}$), CDKN2B ($p15^{ink}$), and CCNG2 (cyclin G2) and down-regulation of genes involved in cell-cycle progression.

ACKNOWLEDGMENTS

The authors thank Dr. Gigi Ostrow from the Gene Expression Core Laboratory of the Interdisciplinary Center of Biotechnology Research, University of Florida for RNA analysis. Thanks are also extended to Kathleen Pennington and Dr. Alan Ealy from the Department of Animal Sciences, University of Florida for providing guidance and access to the real time PCR machine.

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