Rat endometrial *Vdup1* expression: changes related to sensitization for the decidual cell reaction and hormonal control

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Abstract

During implantation in rodents, attachment and invasion of embryonic trophoblast is accompanied by decidualization of the adjacent endometrial stroma. Decidualization can be initiated only when the endometrium is receptive, and this occurs for a short period in pregnancy. The molecular mechanisms underlying this phenomenon remain unclear. In the current study, using differential display and northern blot analysis, we found that steady-state levels of mRNA for vitamin D₃ upregulated protein 1 (*Vdup1*) were significantly higher in 'refractory' and 'delayed' endometrium compared with 'receptive' endometrium or endometrium undergoing artificially induced decidualization. Conversely, thioredoxin (*Txn*), a ubiquitously expressed cellular redox regulator known to promote growth and proliferation, was found to have elevated transcript levels within the decidualizing endometrium. VDUP1 has previously been shown to bind TXN and inhibit its action. In an inverse, but cooperative, relationship, these molecules have been implicated in regulating cell growth and proliferation in a number of tissues and during transformation to cancer. The *Vdup1* mRNA is localized to the uterine stroma in the nonreceptive endometrium, the site of increased *Txn* mRNA levels during decidualization. In addition, *Vdup1* mRNA levels are inversely regulated by progesterone and estrogen; prolonged progesterone exposure stimulates an increase in *Vdup1* mRNA levels whereas estrogen decreases *Vdup1* transcript levels. Together, these results suggest a novel mechanism by which suppression of the decidual response in the nonreceptive endometrium may occur.

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Introduction

Successful implantation depends upon the synchronized maturation of the embryo and the endometrium; the development of the embryo to the expanded blastocyst stage must coincide with hormone-dependent changes within the endometrium that render it receptive to implantation (Psychoyos 1973). Asynchrony between these events can delay or prevent implantation, as the uterus is receptive for only a short period in pregnancy or pseudopregnancy or when the uterus has been prepared with a specific regimen of hormones (Psychoyos 1973, Kennedy & Ross 1997). Interestingly, the nonreceptive uterus is one of the few tissues into which the embryo will not normally implant (Fawcett 1950, Kirby 1963, 1965). While the hormonal requirements for the onset of uterine receptivity in the rodent, as well as those underlying the phenomenon of delayed implantation, have been known in detail for over 30 years (Yochim & De Feo 1963, Psychoyos 1973),

a complete picture of the molecular mechanisms orchestrating the transition from a nonreceptive to a receptive endometrium, and vice versa, remains elusive.

Through the use of mRNA differential display (ddRT-PCR), we exploited both the tight hormonal control for the onset of uterine receptivity and an established model for the induction of a sensitized endometrium in ovariectomized, hormone-treated rats (De Feo 1963, Psychoyos 1973, Kennedy & Ross 1997), and identified vitamin D₃ upregulated protein 1 (*Vdup1*) as a gene upregulated in the nonsensitized rat endometrium. This was the case for both the refractory endometrium (the endometrial state immediately following receptivity) and during a model of delayed implantation, the prereceptive state that supports the survival of an embryo in diapause until sensitization is achieved. VDUP1 has been shown to interact with thioredoxin (TXN) by binding to its active site and inhibiting its biologic action

(Nishiyama *et al.* 1999, Junn *et al.* 2000, Yamanaka *et al.* 2000). TXN has been implicated in numerous cellular processes, including the promotion of cell proliferation, the inhibition of apoptosis and the regulation of transcription factor binding and antioxidant activity (reviewed in Nishiyama *et al.* 2001). In addition, TXN is highly expressed in the uterus of mice (Osborne *et al.* 2001), rats (Sahlin *et al.* 1997) and humans (Perkins *et al.* 1995), as well as in the highly invasive cytotrophoblast cells of the human placenta (Perkins *et al.* 1995). We show that *Txn* mRNA levels increase within the decidualizing endometrium, in contrast to *Vdup1*. The spatial and temporal expression for *Vdup1* and *Txn* mRNA presented in the current study, and the opposing actions of these molecules reported in the literature, suggest that these molecules may interact within the endometrial stroma to modulate the potential for the decidual response in the rat.

Materials and Methods

Animals and tissue preparation

Female Sprague-Dawley rats (200–225 g body mass; Harlan Sprague-Dawley, Indianapolis, IN, USA) were housed in temperature- and light-controlled conditions (14 h light, 10 h dark with lights on from 0500 h to 1900 h) with free access to food and water. Animals were ovariectomized under ether anesthesia (BDH, Toronto, ON, Canada). To obtain rats with uteri differentially sensitized for the decidual cell reaction, estradiol (E_2) and progesterone (P_4) (Sigma) were administered subcutaneously (s.c.) in sesame oil as previously described (Kennedy & Ross 1997). Some rats received a bilateral injection of 100 μ l sesame oil into the uterine lumen on the equivalent of day 5 of pseudopregnancy to induce decidualization (Finn & Keen 1963). Rats receiving intraluminal injections of sesame oil on day 5 and their controls continued to receive daily s.c. injections of E_2 and P_4 in sesame oil until they were killed (Kennedy & Ross 1997). Uteri were collected on the morning of each day of the equivalent of days 4–10 of pseudopregnancy, and, unless stated otherwise, endometrium was separated from myometrium as described by Martel and Psychoyos (1978). All procedures involving animals were performed in accordance with the guidelines of the Canadian Council on Animal Care and the University Council on Animal Care at the University of Western Ontario.

Identification of *Vdup1* by differential display

Changes in endometrial gene expression in endometrium differentially sensitized for decidualization were analyzed by the method of ddRT-PCR as described by Liang and Pardee (1992) and modified by Simmons and Kennedy (2000). In summary, following separation of endometrium from myometrium, it was immediately homogenized in

guanidinium isothiocyanate buffer, and total RNA was extracted (Chomczynski & Sacchi 1987). After DNase I (Gibco-BRL, Burlington, ON, Canada) treatment, total RNA was reversed transcribed using the degenerate 3' primer T₁₁MC, where 'M' is either C, G, T or A. PCR reactions were then performed using the same 3' primer and the 5' primer GATCTGACAC in the presence of [α -³⁵S]dATP (Bauer *et al.* 1993). The PCR products were run on standard 6% (w/v) polyacrylamide-urea sequencing gels, transferred to filter paper, dried and exposed to radiograph film. After development of the film, the apparently differentially expressed cDNA band was cut out of the dried gel, rehydrated, precipitated and then reamplified using the same PCR conditions as for differential display, except that dNTP concentrations were 10-fold higher and [α -³⁵S]dATP was not present. Half of the reamplification products were run in a 1.5% (w/v) agarose gel and stained with ethidium bromide for visualization; the remainder was kept for cloning into the pGEM T-easy vector (Promega). Sequencing was performed using an automated fluorescent DNA sequencer (John P. Roberts Research Institute DNA Sequencing Facility, London, ON, Canada).

northern blot analysis

An amount of 10 μ g of total RNA was denatured and subjected to electrophoresis in a denaturing gel as previously described (Sambrook *et al.* 1989). RNA was then transferred to Hybond-N membrane (Amersham) by capillary transfer and cross-linked by exposure to $1.2 \times 10^5 \mu$ J/cm² of UV energy (Hoefler Pharmacia Biotech, San Francisco, CA, USA). northern blot analysis was performed as described by Church and Gilbert (1984), with some modifications. Briefly, membranes were prehybridized in Church buffer (7% (w/v) SDS, 0.25 mol Na₂HPO₄/l (pH 7.2), 1 mmol EDTA/l and 1% (w/v) BSA) at 65 °C for at least 30 min. The cDNA amplicon isolated from the ddRT-PCR gel was used as a template to synthesize ³²P-labeled DNA probes for northern blot hybridization. An amount of 25 ng of cDNA was labeled by the random-priming technique in the presence of [α -³²P]dCTP (Amersham) with an oligo-labeling kit (Random Primers Labeling System; Gibco-BRL). Hybridizations were carried out at 60 °C for 20 h. The membranes were subsequently washed three times (15 min each) at 65 °C in 20 mmol Na₂HPO₄/l (pH 7.2), with 4% (w/v) SDS and subjected to autoradiography at –70 °C with a Biomax MS TranScreen-HE intensifying screen (Eastman Kodak) for the appropriate exposure time. Blots were then stripped in 1 mmol Tris/l, 1 mmol EDTA/l and 0.1 $\times$ Denhardt's reagent (1 $\times$ Denhardt's: 2% (w/v) BSA, 2% (w/v) Ficoll and 2% (w/v) polyvinylpyrrolidone, pH 8.0) for 2 h at 75 °C, and sequentially reprobed with radiolabeled cDNAs for *Txn*, thioredoxin reductase 1 (*Txnrd1*) and 18S rRNA. The autoradiographs

were digitized using a Hewlett Packard Scanjet 4c/T scanner, and the densitometric intensity of each signal was quantified with Pharmacia ImageMaster VDS Video Documentation System Software, Version 2.0. The 18S rRNA signal was used to determine the relative amounts of RNA loaded into each well and transferred to the membrane and to normalize *Vdup1*, *Txn* and *Txnrd1* signals (Smith & Hammond 1991).

In situ hybridization

At the time total RNA was isolated for the ddRT-PCR and northern blot analyses, some uterine horns were fixed by immersion in 4% (w/v) paraformaldehyde for 24 h. The horns were then rinsed in two changes of PBS and stored in 70% (v/v) ethanol until embedded in paraffin and sectioned at 6 μ m.

The vector, containing an amplicon cloned from the differential display experiments, was linearized and cRNA sense and antisense probes were synthesized in the presence of digoxigenin (DIG)-labeled rUTP (Roche Molecular Biochemicals, Laval, QC, Canada) according to the manufacturer's instructions. The *in situ* hybridization technique used has been described in detail previously (Harvey *et al.* 1995). Briefly, uterine cross-sections were dewaxed in xylene, rehydrated through an alcohol series to 2 $\times$ SSC, digested in proteinase K at 37°C for 5–7.5 min, and acetylated by incubation with freshly prepared 0.1 mol triethanolamine/l and 0.56% (v/v) acetic anhydride at room temperature. After prehybridization at 50°C for 4 h in a 50% (v/v) formamide, 5 $\times$ SSPE, 1 $\times$ Denhardt's solution, the sections were hybridized with 100 ng of cRNA probe and 400 ng of tRNA (in prehybridization solution) under a cover slip for 20 h at 50°C in a sealed humidified chamber. The sections were then washed extensively, treated with RNase and processed for immunologic detection of the DIG-labeled cRNA probe using anti-DIG antibodies at a dilution of 1:500 (Roche Molecular Biochemicals). Color development was allowed to continue until a signal (blue-purple precipitate) was detected. The color reaction for the sense cRNA-treated slides was terminated at the same time as for the antisense slides. Sections were then mounted using GVA-mount (Zymed Laboratories, San Francisco, CA, USA).

Statistical analysis

Experiments quantifying changes in mRNA levels by northern blot analysis were performed three times on separate groups of ovariectomized, hormone-treated rats (except when stated otherwise). The data were analyzed by within-blocks ANOVA, with experiments being considered blocks. Duncan's multiple range test was performed to determine differences between groups. $P < 0.05$ was considered significant.

Results

Identification of *Vdup1* as a gene differentially expressed during the peri-implantation period

Vdup1 was isolated from a differential display screen aimed at identifying changes in gene expression associated with the onset and subsequent disappearance of uterine sensitization for the decidual cell reaction. Specifically, total RNA (pooled from groups of 7 animals) at the equivalent of days 4, 5 and 6 of pseudopregnancy, as well as RNA from two groups of day 5 animals receiving inappropriate doses of E_2 on the evening of day 4 (day 5 'high E_2 ' and day 5 'low E_2 '), were compared. *Vdup1* was identified as a gene upregulated within the day 6, 'refractory' endometrium (Fig. 1). northern blot analysis confirmed the expression pattern seen by ddRT-PCR, as *Vdup1* mRNA levels were significantly higher within the 'refractory' day 6 endometrium than within the 'sensitized' day 5 and 'neutral' day 4 endometrium (Fig. 2, $P < 0.05$). Significantly higher levels of *Vdup1* mRNA were also observed in the endometrium of animals in the day 5, low E_2 group (low E_2 group, Fig. 2, $P < 0.05$). However, rats temporally correct for sensitization (equivalent of day 5 of pseudopregnancy) but with nonsensitized endometrium, as a consequence of a high dose of E_2 on the evening of day 4, had no significant increase in *Vdup1* mRNA levels (high E_2 group, Fig. 2).

Figure 1 mRNA differential display gel showing the cDNA profile for endometrial RNA for the primer combination T11MC (degenerate 3' primer) and GATCTGACAC (5' primer). The total RNA being compared (pooled from the uteri of seven animals for each group) includes endometrial RNA from the equivalent of days 4, 5 or 6 of pseudopregnancy and two endometrial RNA samples from day 5 rats that received inappropriate doses of E_2 on the evening of day 4 (low and high E_2). PCR reactions were run in triplicate, and the black arrow indicates the band representing *Vdup1*.

Figure 2 Profile of *Vdup1* mRNA levels within differentially sensitized rat uteri by northern blot analysis. (A) Endometrium was isolated from the uteri of pseudopregnant animals on the equivalent of day 4, day 5 (maximally sensitized -intermediate E₂) and day 6, and from two groups of animals with temporally correct day 5 uteri that were hormonally nonsensitized (day 5 high E₂ and day 5 low E₂). Total RNA (10 µg), pooled from seven animals per group, was loaded into each lane, and ³²P-labeled probes for *Vdup1* and 18S rRNA were hybridized to the membranes. The experiment was performed three times with independent RNA samples with similar results. (B) Intensities of the signals for *Vdup1* were normalized with 18S rRNA signals, and the average of the three replicates is depicted in the bar graph. Signal intensities from the day 5, intermediate E₂ groups were arbitrarily set at one. Bars with the same superscript are not statistically different from one another.

Northern blot analysis of *Vdup1* and *Txn* mRNA expression during pseudopregnancy and oil-induced decidualization

Further characterization of *Vdup1* mRNA expression from days 6–10 of pseudopregnancy, in the presence ('S') or absence ('N') of artificially induced decidualization, indicated a sustained increase in *Vdup1* transcript levels within the refractory, nondecidualizing endometrium, whereas transcript levels remained low within the decidualizing endometrium (Fig. 3, $P < 0.05$). In contrast, mRNA levels for *Txn* increased during decidualization but remained low within the refractory endometrium (Fig. 3, $P < 0.05$).

Localization of *Vdup1* and *Txn* mRNA expression within the refractory and decidualizing uterus by *in situ* hybridization

In order to elucidate a possible site of action for *Vdup1*, we localized the spatial expression pattern of *Vdup1* mRNA within the day 7 pseudopregnant, 'nonstimulated'

Figure 3 Expression of *Vdup1* mRNA within the rat endometrium during pseudopregnancy as determined by northern blot analysis. (A) Endometrium was isolated from pseudopregnant uteri as described previously. Total RNA was isolated from the endometrium of uteri at the equivalent of days 6–10 of pseudopregnancy. Some animals received an intraluminal injection of 100 µl of sesame oil into the lumen of the uterus ('S' – stimulated) at around noon on the equivalent of day 5 and were killed 1–5 days later. These animals were undergoing artificially induced decidualization at the time of sampling. Those animals that did not receive the intraluminal injection of sesame oil ('N' – nonstimulated) were not undergoing artificially induced decidualization at the time of sampling. Numbers 6–10 refer to the equivalent day of pseudopregnancy. ³²P-labeled probes for *Vdup1*, *Txn* and 18S rRNA were used sequentially in the hybridization. Each lane contained 10 µg of total RNA pooled from seven animals. The experiment was performed three times with independent RNA samples with similar results. (B) Intensities of the signals for *Vdup1* or *Txn* were normalized with 18S rRNA signals, and the averages of the three replicates are depicted in the bar graph. Signal intensities from the day 6, 'stimulated' groups were arbitrarily set at one. Bars with the same superscripts are not statistically different from each other.

and 'stimulated' (decidualizing) endometrium by *in situ* hybridization. *Vdup1* mRNA was highly expressed within the stromal compartment of nondecidualizing endometrium (Fig. 4A). However, by the equivalent of day 7 of pseudopregnancy, 48 h following the application of a decidualogenic stimulus, *Vdup1* mRNA expression was detected in the stromal cells only on the periphery of the endometrium, while expression was absent within the decidualizing stroma immediately surrounding the uterine lumen (Fig. 4E). In contrast, *Txn* mRNA was expressed at low levels within the stroma and luminal epithelium of 'refractory' day 8 pseudopregnant endometrium (with slightly higher levels within the glandular epithelium – Fig. 4C) but was highly elevated within the decidualizing stroma on day 8 of pseudopregnancy (Fig. 4F). No signals were obtained with the sense probes (Fig. 4B and D).

Hormonal control of *Vdup1* and *Txn* mRNA expression in the pseudopregnant uterus

A model of delayed implantation was used to explore further the regulation of *Vdup1* mRNA expression by the ovarian steroids. Ovariectomized rats were given 96 h of

P₄ treatment (after 48 h of P₄ priming, further P₄ treatment in the absence of E₂ results in a 'delayed' endometrium that will become sensitized only after E₂ exposure) followed by either more P₄ ('delayed') or P₄ plus E₂ ('sensitized'). High levels of *Vdup1* mRNA were evident within the uteri of delayed animals, whereas much lower levels of *Vdup1* mRNA were seen in the 'sensitized' animals following the termination of delay (Fig. 5A and B).

The effects of the ovarian steroid hormones E₂ and P₄ on endometrial *Vdup1*, *Txn* and *Txnrd1* mRNA levels were investigated in ovariectomized rats. Animals injected

Figure 4 *In situ* localization of *Vdup1* and *Txn* mRNA within the pseudopregnant rat uterus. Paraffin-embedded uteri were cross-sectioned and analyzed by DIG *in situ* hybridization for *Vdup1* and *Txn* mRNA signals. (A) $\times 10$ magnification, antisense *Vdup1* probe hybridized to 'nonstimulated' day 7 pseudopregnant uteri. (B) $\times 10$ magnification, sense *Vdup1* probe hybridized to 'nonstimulated' day 7 pseudopregnant uteri. (C) $\times 10$ magnification, antisense *Txn* probe hybridized to 'nonstimulated' day 8 pseudopregnant uteri. (D) $\times 10$ magnification, sense *Txn* probe hybridized to 'nonstimulated' day 8 pseudopregnant uteri. (E) $\times 10$ magnification, antisense *Vdup1* probe hybridized to 'stimulated' day 7 pseudopregnant uteri. (F) $\times 10$ magnification, antisense *Txn* probe hybridized to 'stimulated' day 8 pseudopregnant uteri. LE = luminal epithelium, G = glandular epithelium, S = stroma and M = myometrium. Lower magnification panels are taken at $\times 4$ magnification and black bar = 100 μ m for all panels.

Figure 5 Hormonal control of *Vdup1* and *Txn* mRNA levels within the rat uterus *in vivo* as determined by northern blot analysis. (A) Animals were ovariectomized and given 4 mg P₄ daily for 4 days to induce a state of 'delayed implantation', and they then received 4 mg P₄ plus 0.3 μ g E₂ to induce sensitization, or 4 mg P₄ alone to maintain the 'delay'. Animals were killed 18 h later, and total RNA from whole uteri was isolated. There were three animals in each group. In the northern blot analysis, 10 μ g of RNA was used. ³²P-labeled cDNA probes for *Vdup1* and 18S rRNA were used as previously described. (B) Intensity of the signals was normalized with the signals for 18S rRNA (*Vdup1*/18S rRNA), and the averages of the two replicates are depicted in the bar graph. The values for the control groups were arbitrarily set at 1. (C) Animals were ovariectomized and given a single dose of either 1 μ g E₂, 4 mg P₄, 1 μ g E₂ plus 4 mg P₄, or vehicle (sesame oil). Uteri were collected 18 h later, and total RNA from whole uterus was isolated. There were three animals in each group. In the northern blot analysis, 10 μ g of RNA was used. ³²P-labeled cDNA probes for *Vdup1*, *Txn* and 18S rRNA were used for hybridization. (D) Intensity of the signals was normalized with the signals for 18S rRNA (*Vdup1*/18S rRNA), and the averages of the two replicates are depicted in the bar graph. The values for the control groups were arbitrarily set at 1.

with a single dose of E₂ showed a significant decrease in *Vdup1* mRNA levels while *Txn* mRNA levels were not significantly altered (Fig. 5C and D). P₄ alone or P₄ plus E₂ in combination did not significantly alter expression of either *Vdup1* or *Txn* mRNA levels over that of control, although it eliminated the E₂-induced decrease in *Vdup1* mRNA levels (Fig. 5C and D). *Txnrd1* mRNA levels were significantly increased by treatment with E₂ alone.

Discussion

Using differential display to uncover changes in gene expression associated with the onset and subsequent disappearance of uterine sensitization for the decidual cell reaction, the current study has identified *Vdup1* as a gene upregulated within the 'refractory' endometrium (Fig. 1). Subsequent northern blot analysis confirmed the increase in endometrial *Vdup1* mRNA levels following uterine sensitization (Fig. 2) and also indicated that this increase is sustained as long as the endometrium remains in a 'refractory' state (Fig. 3). In contrast, endometrial *Vdup1* mRNA levels are not elevated following sensitization if decidualization has been initiated (Fig. 3). *In situ* hybridization experiments localized the site of *Vdup1* mRNA expression to the stromal cells of the endometrium (Fig. 4). Together, these data suggest a possible role for VDUP1 in maintaining stromal cells of the endometrium in a refractory state.

Vdup1 was originally discovered as a gene upregulated during 1,25-dihydroxyvitamin D₃-induced differentiation of HL-60 cells (Chen & DeLuca 1994) and downregulated in *N*-methyl-*N*-nitrosourea-induced mammary tumors (Young *et al.* 1996), suggesting that VDUP1 may be involved in the regulation of cell growth and tumor suppression. The inference that VDUP1 functions as a negative regulator of cell growth and in tumor suppression has gained support recently, as a number of studies have shown a decrease in *Vdup1* gene expression associated with the onset of various cancers, including breast (Butler *et al.* 2002), colon (Butler *et al.* 2002, Ikarashi *et al.* 2002) and B-cell lymphoma (de Vos *et al.* 2003). In addition, some studies have shown that an increase in VDUP1 protects against cancer and inhibits tumor cell growth (Butler *et al.* 2002, Goldberg *et al.* 2003, Han *et al.* 2003).

VDUP1 has also been implicated as a binding protein for the redox regulator TXN, binding to its active site and inhibiting its biologic activity (Nishiyama *et al.* 1999, Junn *et al.* 2000, Yamanaka *et al.* 2000). TXN has been shown to have a wide range of biologic activities, including growth promotion and proliferation, antioxidant activity, regulation of transcription factor binding and inhibition of apoptosis (reviewed in Nishiyama *et al.* 2001). It is possible that the growth inhibition effects of VDUP1 are mediated through TXN inhibition. In fact, Schulze *et al.* (2002) recently described a system in which VDUP1 and TXN have opposing effects on human aortic smooth muscle cell proliferation. Specifically, overexpression of

VDUP1 inhibited cell proliferation, in contrast to TXN-stimulated cell proliferation, as well as prevented nuclear translocation of TXN in response to platelet-derived growth factor, a potent stimulator of aortic smooth muscle cell proliferation and TXN activity (Schulze *et al.* 2002).

Because VDUP1 has been shown to regulate cell proliferation and growth negatively through inhibition of TXN activity, and because TXN has been shown to be highly expressed in the uterus of mice (Osborne *et al.* 2001) and humans (Perkins *et al.* 1995), as well as in the highly invasive cytotrophoblast cells of the human placenta (Perkins *et al.* 1995), we investigated whether *Txn* mRNA levels would have an opposite expression pattern to that of *Vdup1* in the rat endometrium. Indeed, *Txn* mRNA levels increased within the decidualizing endometrium but remained low within the nondecidualizing, refractory endometrium (Fig. 3). While *Vdup1* and *Txn* mRNA levels within the endometrium appear to have opposing expression patterns, they are both induced in the endometrial stroma (Fig. 4). This converse temporal expression but shared spatial expression suggests that they may have linked, but opposing roles in stromal cell proliferation within the uterus, with TXN stimulating the proliferation of decidual cells and VDUP1 inhibiting cell proliferation in the refractory or nonsensitized endometrial stromal compartment.

In addition to verifying the results obtained by ddRT-PCR, northern blot analysis also revealed a statistically significant increase in *Vdup1* mRNA levels within the endometrium of animals in the day 5, low E₂ group (low E₂ group, Fig. 2). These animals, although temporally correct for sensitization (equivalent of day 5 of pseudopregnancy), were hormonally nonsensitized, as they did not receive E₂ on the evening of day 4 but only an injection of P₄. By contrast, rats with nonsensitized endometrium on day 5, as a consequence of a high dose of E₂ on the evening of day 4, had low levels of *Vdup1* mRNA (high E₂ group, Fig. 2). These data would indicate that prolonged P₄ exposure in the absence of E₂, a situation that simulates lactational delay of sensitization/receptivity, results in an increase in *Vdup1* mRNA levels. To investigate this observation further, we used a model of delayed implantation. Ovariectomized rats were given 96 h of P₄ treatment (after 48 h of P₄ priming, further P₄ treatment in the absence of E₂ results in a 'delayed' endometrium that will become sensitized only after E₂ exposure) followed by either more P₄ ('delayed') or P₄ plus E₂ ('sensitized'). High levels of *Vdup1* mRNA were evident within the uteri of delayed animals, whereas low levels of *Vdup1* mRNA were seen in the 'sensitized' animals following the termination of delay (Fig. 5A and B). The fact that *Vdup1* mRNA is upregulated within the endometrium during 'delayed implantation' further supports the hypothesis that VDUP1 may help to maintain a quiescent, nondecidualizing stromal cell population. In addition, E₂ administration, which is itself a known promoter of cell growth, is sufficient significantly to downregulate *Vdup1* mRNA levels within the

uteri of ovariectomized animals (Fig. 5C and D). The absence of an E₂-induced increase in *Txn* mRNA (Fig. 5C and D) was unexpected, as Sahlin *et al.* (1997) showed an increase in *Txn* mRNA within the rat uterus in response to E₂, although this could be due to the fact that in their study 2.5 µg of E₂ was injected, whereas we used only 1.0 µg. We did see, however, a significant increase in *Txnrd1* mRNA levels in response to 1.0 µg of E₂ (Fig. 5C and D). TXNRD1 is a protein that reduces the oxidized, inactive form of TXN back to its active state (Holmgren & Bjornstedt 1995). Presumably, an increase in *Txnrd1* expression would translate into an increase in *Txn* activity within the cell. Together these data indicate that E₂ decreases *Vdup1* mRNA levels and possibly increases TXN activity through decreased TXN inhibition and increased TXN recycling, while P₄ prevents the E₂-induced changes in gene expression. P₄ alone does not induce an increase in *Vdup1* mRNA levels, but, rather, a prolonged exposure to P₄, as in the case of 'delayed implantation', is required (Fig. 5A and B).

The expression patterns of *Vdup1* and *Txn* mRNA within the rat uterus presented in the current study, along with the established interaction of these proteins to regulate cell proliferation in other systems, positions these molecules as potential regulators of cell proliferation in the rat uterus around the time of embryo implantation. While the function of VDUP1 in the nonsensitized endometrium is probably the inhibition of TXN, this has yet to be established experimentally. Other TXN-independent functions may exist for VDUP1. In fact, a recent study by Han *et al.* (2003) showed that VDUP1 induced cell-cycle arrest and physically interacted with transcriptional corepressors, presumably inhibiting cell growth in a TXN-independent fashion. Clearly, more work is needed to clarify the function of VDUP1 within the endometrium during the peri-implantation period.

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