Large scale validation of human N-myc Downstream-Regulated Gene (NDRG)-1 expression in endometrium during the menstrual cycle

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A major challenge in the comprehension of the endometrial transformations leading to the completion of each menstrual cycle in humans is in the identification of specific molecular pathways underlying these monthly turnovers. Towards this goal we compared, by the differential display technique, the relative expression of mRNA in endometrial biopsies harvested in individuals (n = 48) either at the proliferative or the secretory phase of the menstrual cycle. We isolated a cDNA fragment homologous to NDRG1 (N-myc Downstream-Regulated Gene-1) that is present in markedly higher amounts in the secretory phase. Northern blot analysis and quantitative real time PCR experiments confirmed this result in distinct cohorts of individuals (44 and 560 respectively). A closer examination of data showed that the highest mRNA levels were found during the range of 25−28 days of the uterine cycle. Consistent with the mRNA data, the temporal profile of the NDRG1 protein showed a 15-fold increase during the secretory phase, as demonstrated by using semi-quantitative dot blot analyses (n = 92). Immunohistochemical localization revealed that NDRG1 was expressed both in epithelial and stromal cells. This large scale validation of the NDRG1 mRNA and protein increase in endometrium during the secretory phase is consistent with its differentiation-related function described in other tissues and its potential involvement in the window of implantation of the human endometrium, as suggested by previous chip-based evidence.

Key words: endometrium/menstrual cycle/NDRG1

Introduction

The cyclic growth, differentiation and breakdown of the human endometrium represent perhaps the most dynamic example of steroid-driven tissue plasticity in the adult. The endometrial tissue is composed of epithelial cells and connective tissue called stroma. During the proliferative (P) phase of the menstrual cycle (days 1−14), different cell types in the endometrium coordinately undergo extensive growth in response to the synergistic action of estrogens, growth factors and cytokines that leads to ovulation (Wang and Chard, 1999; Bigsby, 2002). This temporal checkpoint initiates a global tissue maturation and differentiation process, taking place in the secretory (S) phase (days 14−28), mainly governed by progesterone. During the S-phase, the functionalis epithelium thickens, epithelial cells elongate and ciliogenesis takes places both in epithelial and surface cells (Campbell et al., 2000). This rapid steroid-mediated tissue development, which rivals that of many neoplasias, allows blastocyst nidation during a specific time-frame called the ‘window of implantation’ at days 20−24, at the progesterone secretion peak (Lessey et al., 1998; Giudice et al., 1999). In the absence of implantation and the continued progestational environment of pregnancy, the superficial functionalis layer of the endometrium loses its receptive state, undergoes degradation, and is expelled with menstrual blood flow (Salamonsen et al., 1999). Within a period of 5−6 days, the entire lining regenerates without scarring, constituting a remarkable example of controlled tissue remodelling.

A complex cascade of interplaying genes governs the entire menstrual cycle process. Many steroid-induced endometrial genes have been identified and their expression pattern during the menstrual cycle has been characterized in vivo using various approaches (Rodgers et al., 1994; Apparao et al., 2001). Some endometrial transcripts are directly regulated by progesterone during the S-phase, e.g. glycodulin, insulin-like growth factor-1 (IGFBP-1) and tissue inhibitor of metalloproteinase-3 (TIMP-3) (Dong et al., 2002; Tseng et al., 2002). Given the spectacular impact of progesterone on tissue remodelling during the S-phase and its downstream consequences on critical processes such as fertility, identification and characterization of molecular intermediates of this specific cascade are of great interest. To this end, we carried out a comparative gene expression profiling study between the P- and S-phases of the menstrual cycle in order to identify new subsets of genes modulated during the peak of progesterone, occurring between days 17−25 (Tabibzadeh et al., 1998; Nikas et al., 2000). We chose to use a screening approach, differential display (DD), which allows for analysis of individual, and simultaneously several, samples. We focus herein on the identification and characterization of a cDNA fragment, namely DD5, that was present in much higher concentrations in S-phase samples. Nucleotide sequence of DD5 revealed that this transcript was identical to that of N-myc Down-Regulated Gene-1 (NDRG1), also termed RTP, CAP 43, rit42, TDD, or PROXY-1 in the literature. This observation was confirmed by northern blot analysis and a validation was performed on a large cohort of samples by real time PCR, allowing a more precise profiling of the gene. Levels of the NDRG1 protein were also shown to be modulated during the menstrual cycle, in epithelial as well as stromal cell types. Finally, mapping of the NDRG1 protein was...
performed on endometrial tissues by immunohistochemistry. This study constitutes a comprehensive temporal analysis of NDRG1 steady state mRNA and protein levels in endometrium collected from a large population of normally ovulating women.

### Materials and methods

#### Patient recruitment

Women who provided endometrial biopsies for this study were undergoing laparoscopy for various medical indications such as tubal ligation, tubal reanastomosis, hysterectomy and invasive surgery. The inclusion criteria of the study were the following: pre-menopausal age; not currently menstruating; menstrual cycles between 21 and 35 days in length; no acute salpingitis; no prior HIV diagnosis; no prior hepatitis B or C infection; not currently pregnant or in the last 3 months; not currently breastfeeding; no current use of a compound selected from the group consisting of GnRH agonists, progestins, Danazol, and oral contraceptives, or in the last 3 months; no current use of intrauterine device or in the last 3 months.

The participants in the study were subdivided into P- and S-phase groups based on the phase of the menstrual cycle indicated by the date of their last menstruation and confirmed by histological examination from a section of formalin-fixed endometrial tissue (Fox, 1995). Subjects ranged between 29 and 38 years of age. No statistical bias due to age or primary medical indication for surgery was observed between the P- and S-phase groups (data not shown). Different subgroups were used for DD and northern experiments, while all samples were analysed by real time PCR. This study was approved by PROCREA Biosciences Ethics Review Board and Internal Review Board of each participating clinical institution. Endometrial biopsies were obtained from women who gave informed written consent.

#### Isolation and preparation of RNA from biopsies

Endometrial biopsies (n = 560) were performed with Pipelle catheters under sterile conditions, from the uterine fundus. A portion of each biopsy was transferred in RNA later® (Ambion, Inc., USA), maintained on ice during transport from the hospital to our laboratory, and then stored at −80°C until RNA extraction. Total cellular RNA was isolated directly from endometrial tissue by extracting RNA from 24 P- and 24 S-phase endometrial biopsies. Total RNA samples, extracted from 24 P- and 24 S-phase endometrial biopsies. Brieﬂy, first strand cDNA synthesis was performed using 200 ng RNA in the presence of 0.01 mol/l dithiothreitol (DTT), 20 mol/l/dTTP, 0.2 mol/l of anchor primers H-T11-M (where M is G or A or C; Genset Corp, USA) and 200 IU of M-MLV reverse transcriptase (RT) in the appropriate reaction buffer (Invitrogen Life Technologies, Canada). RT reactions were performed in a total volume of 20 μl at 65°C for 5 min, followed by 37°C for 1 h, and 5 min at 75°C. S-phase-specific DD PCR products were isolated by subjecting 2 μl of the RT reaction to PCR amplification in the appropriate buffer containing 2 μmol/l dNTP, 1 IU /μl DNA Polymerase and [γ-32P]dATP (2500 Ci/mmol) (all from Amersham Pharmacia Biotech) and 0.2 μmol/l of anchor and arbitrary primers (Genset Corp). A total of 18 different combinations of primer pairs (three anchor primers and six arbitrary primers) were used for the PCR reactions. Amplifications were performed in a Stratagene Robocycler and involved 40 cycles of 94°C (1 min), 40°C (2 min) and 72°C (1 min) with a final extension of 7 min at 72°C.

Equivalent amount of DNA samples (based on levels of GAPDH expression determined by RT–PCR using specific primers, data not shown) were electrophoresed on a 6% denaturing polyacrylamide gel, and the dried gels were exposed to film. Differentially modulated bands (between P- and S-phase samples) were excised from the gel and DNA was reamplified according to the same PCR reactions described above and cloned using the TA Cloning® Kit (Invitrogen Life Technologies) according to the manufacturer’s instructions. Clones containing the desired fragments were identified by colony-PCR, at least three colonies per CDNA candidate were selected and validated by reverse northern blotting to decrease as much as possible the number of false positive clones. Brieﬂy, this consisted of spotting PCR-ampliﬁed DNA fragments puriﬁed from the DD gels onto two identical nylon membranes (Boehringer Ingelheim Ltd, Canada) and then hybridized with two 32P-labelled DD PCR reactions, one from each phase, as probes. Still differential signals were kept in the study for the next step. Again, three clones per candidate with the expected differential pattern were selected for nucleotide sequence determination, performed using the ABI Prism Rhodamine cycle sequencing kit™ and run on an automated ABI Prism 310™ Sequencer (Applied Biosystems Canada). Nucleotide sequence alignments were performed with Genbank™ through the BLAST software available at the National Center for Biotechnology Information (NCBI) website (www.ncbi.nih.gov).

#### Northern blot analysis

Northern blots were performed according to standard protocols (Sambrook et al., 2001). Brieﬂy, 10 μg total RNA samples were resolved on formaldehyde gel and transferred onto Hybond N+ membranes (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. RNA samples were immobilized on the membranes by UV irradiation and blots were hybridized overnight at 42°C in ULTRAhyb (Ambion, Inc.) with specific probes corresponding to cDNA fragments of NDRG1 coding regions (GenBank accession no. NM_0006096; nucleotide region: 2726–2939) and 18S rRNA (GenBank accession no. X03205; nucleotide region: 613–980) as a loading control. Identity of the ampliﬁed cDNA probes was assured by nucleotide sequence determination and fragments were then cloned in pCR®2.1 (TA Cloning® Kit; Invitrogen Life Technologies) and 32P-labelled using a random primer labelling kit (Strip-EZ DNA, Ambion, Inc.). Hybridization signals were visualized by phosphorimaging, and quantiﬁcation of the bands was performed using Gel-Pro® Analyser (version 4.0 software). Background-adjusted band intensities were normalized based on the intensity of 18S rRNA.

#### Quantitative real time RT–PCR

Total cellular RNA, previously isolated from endometrial biopsies as described above, was used as a template for the production of cDNA. Brieﬂy, 2 μg of DNA was digested in a total of 20 μl with 1 IU DNase I (Amplitication Grade, Invitrogen Life Technologies) in 20 mol/l Tris–HCl (pH 8.4), 2 mol/l MgCl2, 50 mol/l KCl at room temperature for 5 min, then at 65°C for 10 min. The reaction was terminated with 2.5 mmol/l EDTA. DNAase-treated RNA was then reverse-transcribed into cDNA in a total of 50 μl using 400 IU M-MLV reverse transcriptase in the presence of 100 ng random primers, 10 mmol/l DTT, 0.5 mmol/l (final concentration) of each of dGTP, dATP, dTTP and dCTP (all from Invitrogen Life Technologies). Following incubation at 37°C for 1 h, heating to 70°C in the presence of 1 mmol/l EDTA for 15 min terminated the reaction.

#### Table I. Oligonucleotide primers used in real-time PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>GenBank</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Fluorogenic probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDRG1</td>
<td>NM006096</td>
<td>CATCGGAGATGAACCACAAAA</td>
<td>TGCTGGGTGATCTCTGCTGCA</td>
<td>CTGCTACAACCCCTCCTCAACTACGGG</td>
</tr>
<tr>
<td>CK8</td>
<td>M26324</td>
<td>TCCAGATCTTGAGACACATCT</td>
<td>GGAGCCGGGGTTGTTGCTCAT</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>

[2.1 (TA Cloning® Kit; Invitrogen Life Technologies) and then hybridized with two 32P-labelled DD PCR reactions, one from each phase, as probes. Still differential signals were kept in the study for the next step. Again, three clones per candidate with the expected differential pattern were selected for nucleotide sequence determination, performed using the ABI Prism Rhodamine cycle sequencing kit™ and run on an automated ABI Prism 310™ Sequencer (Applied Biosystems Canada). Nucleotide sequence alignments were performed with Genbank™ through the BLAST software available at the National Center for Biotechnology Information (NCBI) website (www.ncbi.nih.gov).]
Figure 1. Representative differential display (DD) analysis of proliferative (P-) and secretory (S-) phase endometrial biopsy samples. The arrow indicates the up-regulated PCR fragment in S-phase samples, namely DD5 homologous to NDRG1.

A relative standard curve (SC) for real time PCR, representing six 6-fold dilutions of a pool of cDNA, was used as a common set of samples that linked the 16 experimental PCR plates together and permitted the overall analysis of 560 samples (see Statistical data analysis below). Preparation and utilization of this SC as a quality control of the efficiency of amplification of the PCR plate is described elsewhere (Merlen et al., submitted for publication).

Real time PCR reactions were performed using TaqMan® chemistry for NDRG1 and SYBR Green chemistry for CK8 (Applied Biosystems Canada). PCR primers and fluorogenic probe were designed using Primer Express, version 1.5 (Applied Biosystems Canada) to amplify unique sequences in each gene of interest and are presented in Table I.

Fluorogenic probe was labelled with the reporter dye 6-carboxyfluorescein (6’-FAM) at the 5’ end and with the quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3’ end. In order to exclude genomic DNA contamination during the amplification of NDRG1, the amplicons spanned at least one exon-exon boundary. The CK8 gene has one single exon, making a similar strategy impossible.

Large scale population study of real time PCR reactions involved eight 96-well plates per phase of the menstrual cycle, whereby each plate contained duplicates of each dilution of the SC, duplicates of a no-template control, and duplicates of the 560 endometrial samples.

Quantitative–competitive (QC) algorithm and statistical data analysis
Details of the algorithm used for real time PCR data analysis are described elsewhere (Merlen et al., submitted for publication). Cycle threshold (CT) values were directly used as a measure of gene expression, without conversion in relative copy numbers. Final results (ACΔCT) were transformed from exponential to linear space through 2−ΔΔCT calculation method (ABI user bulletin 2, 1997). CT value is inversely proportional to the initial concentration of a given mRNA, since the higher the level of expression, the lower the number of PCR cycles needed to reach the threshold value of detection of the fluorescent signal.

All CT values >35 were excluded from the study because of the accuracy limits of the PCR machine. Briefly, data from each plate is analysed for experimental errors on duplicates and for detection of ‘bad runs’ (Tukey’s mean-difference plot). In a second step, all plates were assembled, standard curves as well as sample data were compiled in two series of data and analysed in parallel for inter-plate variations and potential statistical bias in the population distribution.

Quality-controlled data were then submitted to statistical comparison tests according to their distribution. In this report, all distributions were normal, allowing the use of Student’s t-tests for the statistical analysis. SPSS and Excel softwares were used for statistical analysis. For all analyses, P < 0.0001 was considered significant. Box plot graphs show the median (line within the box), quartiles (50% of the samples are located within the box), outliers (open circles) and extreme values (*). Outliers are between 1.5 box length and 3 box lengths from the box. Extremes are >3 box lengths from each extremity of the box. Whisker caps represent the maximum/minimum values of the population.

Isolation and preparation of proteins from endometrial biopsies
Total cellular protein extracts were isolated directly from endometrial biopsies (n = 92). Briefly, 200–300 mg of biopsy tissue was homogenized at room temperature using an electric homogenizer in the presence of 3 ml of lysis buffer consisting of 20 mmol/l Tris–HCl (pH 7.5), 0.1 mol/l NaCl, 2% sodium dodecyl sulphate, 5 mmol/l EDTA, 0.5 μg/ml leupeptin (Boehringer Ingelheim Ltd), 2 μg/ml aprotinin, and 200 μg/ml phenylmethylsulphonylfluoride (both from Sigma–Aldrich, Canada), lysed for 10 min at 100°C and centrifuged for 20 min at 14 000 g. Protein determination of supernatants was made using the DC Protein Assay™ (Bio-Rad Laboratories Ltd, Canada) according to the manufacturer’s instructions.

Protein dot blotting
Protein dot blot experiments were performed on protein extracts obtained from biopsies (n = 32, P-phase; n = 60 S-phase). Samples were spotted in duplicate using 2.5 and 5 μg of protein in order to avoid saturated signals. Spotting was performed on Hybond™ ECL™ nitrocellulose membranes (Amersham Pharmacia Biotech). The membranes were blocked in 2.5% dry milk powder in TST buffer (10 mmol/l Tris–HCl pH 7.5 containing 100 mmol/l NaCl and 0.1% Tween-20), and incubated in a 1:2000 dilution (in 5% milk–TST) of either the rabbit polyclonal anti-NDRG1 antibody (Skuld-Tech) or a goat polyclonal anti-actin antibody (Santa Cruz Biotechnology) for 1 h. The anti-NDRG1 antibody used in this study was raised against a synthetic peptide mimicking the Ni/Cu2+ binding C-terminal sequence of the protein specific for the NDRG1 transcript (Piquemal et al., 1999; Zoroddu et al., 2001). Membranes were then incubated with a 1:2000 dilution of either a horseradish peroxidase (HRP)-conjugated goat anti-rabbit polyclonal antibody (Pharmingen) or a HRP-conjugated mouse anti-goat polyclonal antibody (Santa Cruz Biotechnology). After 1 h incubation with the secondary antibodies, membranes were washed and revealed by ECL™ chemiluminescent system according to the manufacturer’s protocol (Amersham Pharmacia Biotech). Membranes were exposed to Biomax MS films (Kodak). Multiple exposures of the films at variable time-points were done in order to capture an appropriate unsaturated signal intensity of the proteins of interest, and the most accurate ones were selected for densitometry analysis via Molecular Analyst software (Bio-Rad Laboratories). Incubations of the membranes with either non-immune rabbit IgG (Skuld-tech) or secondary antibodies alone were performed as negative controls; no signal was detected in either case (results not shown).

To diminish experimental variation, mean values were calculated between duplicate spots for those in which the coefficient of variation was <20%; this threshold value of 20% was selected based on a 95% confidence interval criteria. A SC generated by a pool of biopsy protein extract, representative of both phases of the menstrual cycle, and serially diluted in duplicate spots containing 28, 14, 7, 3.5, 1.75 and 0.875 μg of protein, was spotted on each blot (see schematic membrane setup in Figure 4A). This SC was used for two purposes: (i) to transform OD units to μg of protein using the curve regression (see representative examples in Figure 4B), and (ii) to determine the linear zone of OD readings for each autoradiogram. By this means, only OD readings within the range of 0–20 units were used for our analyses and were found globally to represent 95% of our measurements. Finally, quantification of expression levels was performed on absolute amounts of proteins quantified as described in the previous section.

Immunohistochemistry
For these experiments, S-phase endometrial biopsies were snap-frozen at −80°C upon isolation. Slides with serial cryosections were fixed in acetone for 3 min at 4°C and then blocked in 10% normal goat serum/PBS for 5 min at 25°C (Dako Diagnostics Canada), washed in PBS, and incubated 2 h at 25°C with either a rabbit polyclonal anti-NDRG1 antibody (Piquemal et al., 1999; Skuld-Tech, Université Montpelier II, France); a mouse anti-human epithelial antigen (clone Ber-EP4 from Dako) or a mouse anti-vimentin (Dako). All antibodies were diluted in 10% normal goat serum/PBS. Slides were washed with PBS prior to incubation (2 h) with the appropriate ALEXA fluor® 546-labelled secondary antibodies (Molecular Probes, USA), and counterstaining with DAPI III (Vysis, USA). Secondary antibodies used alone as negative controls did not yield any staining (results not shown).

Results
Identification of a DD signal (an up-regulated DD fragment) in S-phase biopsies
Total RNA isolated from individual (n = 24 in each phase) endometrial biopsies were subjected to DD assays in order to identify
gene products that would discriminate between the P- and S-phases of the menstrual cycle. A representative DD autoradiogram of PCR products showing the presence of DD5 as a clear differential signal in a subset of six proliferative and six secretory phase biopsies is presented in Figure 1. Complementary DNA sequence determination performed on three independent clones revealed that DD5 was homologous to the 3' end of NDRG1 (GenBank accession NM006096).

Confirmation of the DD observation by northern blotting
To confirm the DD RT–PCR results, we first measured mRNA levels of NDRG1 throughout the menstrual cycle using northern blot analyses. Total RNA samples were isolated from 44 biopsies equally distributed in the P- and S-phases. The experiment was performed with a different cohort from that used for DD. RNA samples were sequentially hybridized with either NDRG1 or 18S rRNA probes (see Figure 2A). The P- and S-phase populations had a median dating (± SD) of 12 ± 2 and 24 ± 1 days respectively. Results from the densitometry analysis are presented in box plots in Figure 2B. Comparable mRNA levels between P- and S-phases were observed for 18S rRNA (1.12-fold ratio) resulting in a non-significant t-test comparison ($P = 0.46$), allowing its use as a loading control for the comparison of S- and P-phase endometrial RNA samples. By using 18S rRNA as a normalizer, the NDRG1 transcript is shown to be present in significantly higher amounts ($P < 0.0001$) by 5.2-fold in the S-phase.

Validation of NDRG1 S-phase-specific increase by real time PCR
Real time RT–PCR was used on a large scale population of endometrial biopsy samples ($n = 560$, 280 in each phase) to validate NDRG1 modulation throughout the menstrual cycle. For this purpose, we have previously developed a quality control procedure (see Materials and methods section) enabling us to assemble data from samples run on several 96-well plates. PCR data from NDRG1 mRNA are presented in Figure 3A.

To assess the cellular heterogeneity of endometrial tissue samples, we measured in parallel mRNA levels of an epithelial marker, cytokeratin 8 (CK8). Results showed similar levels of mRNA in the two populations for this gene, suggesting certain homogeneity in the...
cell content of the biopsy samples (Figure 3B). In addition, we used CK8 as a normalizer of the NDRG1 data, since the high abundancy of 18S rRNA (northern normalizer) made its use impossible in the PCR-based approach. CK8 PCR data were also submitted to the QC process, and normalization of NDRG1 data was finally carried out for each sample (Figure 3C). Higher NDRG1 mRNA levels in the S-phase is reflected by lower CT values. Fold modulation between the S- and P-phase populations was calculated according to $2^{(-\Delta\Delta CT)}$, where $\Delta CT = (\text{mean CT}_{S\text{-phase}}) - (\text{mean CT}_{P\text{-phase}})$ (ABI’s User Bulletin 2, 1997). NDRG1 mRNA levels are significantly higher in the S-phase, displaying a fold modulation of $\times3.5$, when normalized with CK8 ($P < 0.001$, Figure 3C).

With a high number of samples, it became possible to subdivide groups according to the day of the menstrual cycle in which each biopsy sample had been collected. This allowed us to monitor more precisely NDRG1 mRNA concentrations. Since no accurate histological dating is possible for the P-phase, only the S-phase samples were subdivided in groups of 5 days for the two first (15–19 and 20–24) and of 4 days (25–28) for the third sub-category of the S-phase. Such an analysis based on CT mean values of each group (Figure 3D) showed that this specific mRNA increased along the S-phase, with highest levels reached in the last sub-category (25–28 days) just before the onset of menses.

Western blot analysis of NDRG1 protein expression in endometrial biopsy samples

To complete NDRG1 profiling in the endometrium, protein levels were examined in biopsies isolated throughout the menstrual cycle. We analysed the temporal levels of the NDRG1 protein during the menstrual cycle using the same anti-NDRG1 polyclonal antibody. This antibody is directed against the specific Ni/Cu$^{2+}$ binding C-terminal sequence of NDRG1 (Piquemal et al., 1999) and recognizes a
single protein of the expected 43 kDa mass in Western blot analysis of protein extracts of endometrial biopsies (data not shown). The population used in the semi-quantitative dot blot study, i.e. 32 individuals in the P-phase and 60 in the S-phase, has a median dating value (± SD) of 13 ± 6 and 21 ± 4 days respectively. Performing such a study using standard 96-well equipment required the establishment of a quantitative dot blot platform allowing inter-blot comparison. Our approach, in an enzyme-linked immunosorbent
assay-like detection set-up (see Figure 4A for a schematized illustration), used two sets (2.5 and 5 μg) of duplicate samples, which were immunoblotted along with a serial dilution set of samples used as reference in duplicate points, generating a SC for each membrane (three representative SC regressions are shown in Figure 4B). Figure 4C shows a representative subset of immunode-tected dot blots in which 59 biopsy samples were analysed for NDRG1 quantification. The box plot graph in Figure 4D summarizes NDRG1 protein quantification in S- and P-phase biopsy samples (2.5 μg spots) using transformed OD readings. Numbers on the top of each box represent the number of samples matching all QC criteria and used for the evaluation of the mean values and fold modulations. Two analyses were performed using data obtained with 2.5 and 5 μg spots and similar results were found for NDRG1 with both series of results (data not shown), revealing a statistically significant increase \( (P < 0.0001) \) from P- to S-phase of 19-fold between the mean values of the two sample groups compared.

**Protein localization in the endometrial tissue**

Serial cryosections of S-phase endometrial biopsies were processed for immunohistochemistry using antibodies specific for endometrial glandular and stromal cells as localization controls (Ber-EP4 and anti-vimentin, respectively; Tabibzadeh, 1991) as well as the anti-NDRG1 polyclonal antibody. Figure 5 shows the results of two independent immunostainings (slides 1 and 2). Ber-EP4 staining is intense in epithelial cells of endometrial glands (note that identical structures on each slide are indicated by ‘G’) and lack of staining for the stroma (indicated by ‘S’). A reverse pattern is observed in the next section, when using anti-vimentin antibody as a stromal cell marker. Alternatively, both cell types were stained using the anti-NDRG1, suggesting that this protein is ubiquitously expressed in the endometrium, although a higher staining for epithelial cell type is visible.

**Discussion**

This report represents the first comprehensive study of human NDRG1 temporal expression profile, both at steady state mRNA and protein levels, in the endometrium throughout the menstrual cycle. Given the significant change in mRNA as well as protein steady state levels around ovulation (day 14), and the fact that at this temporal window radical hormonal changes take place in endometrium, these results strongly suggest a de-novo sex steroid-dependent induction of expression. We found a significant S/P modulation of NDRG1 mRNA of 5.2-fold \( (P < 0.0001) \) using northern blot analysis when normalized with 18S rRNA, and S/P modulation of 3.5-fold \( (P < 0.0001) \) using real time PCR when normalized with CK8. The tissue staining with the anti-NDRG1 antibody suggests the presence of higher levels of this protein in epithelial cells. Nevertheless, the fact that the CK8 levels are quite stable between the two phases strongly supports the idea of an actual up-regulation of expression rather than a larger number of epithelial cells in the S-phase.

These data are consistent with two other gene profiling studies in the human endometrium using different experimental designs and analysis algorithms, and reporting on 3.2- and 3.5-fold S/P modulation of NDRG1 (RTP) transcript using Affymetrix oligoarrays (Kao et al., 2002; Borthwick et al., 2003).

NDRG1 belongs to the recently identified NDRG family that contains four members, namely NDRG1–4, encoded by four distinct genes (Okuda and Kondoh, 1999; Zhou et al., 1998; Qu et al., 2002). NDRG1 was shown to respond to various inducers such as homocysteine in human endothelial (Kokame et al., 1996) and colon cells (Van et al., 1997), to hypoxia in several cell types (Salnikow et al., 2000b), and to nickel and cobalt mimicking hypoxia in lung cells (Zhou et al., 1998; Salnikow et al., 2000a). As a potential differentiation marker, this gene is down-regulated in transformed cell lines, as compared with their normal counterparts (Park et al., 2000; Cangul et al., 2002; Qu et al., 2002), and its expression was shown to be modulated both in vitro and in vivo by N-myc, P53 and hypoxia-
inducible factor (HIF)-α (Kurdistani et al., 1998; Okuda and Kondoh, 1999). More specifically, steroid hormones were described among potential inducers of this gene. Estrogen and androgen responsiveness have been reported in breast and prostate cancer cell lines; for example, anti-estrogen ICI182 induces NDRG1 mRNA (Piquemal et al., 1999), whereas testosterone and dihydrotestosterone down-regulate its expression (Lin and Chang, 1997).

No clear function of NDRG1 has been demonstrated in the tissues where this gene is expressed and identification of its endogenous regulators in the human endometrium transcends the goal of the current investigation. Nevertheless, it has been shown that distinct signalling pathways modulate its transcription, mRNA stability, translation and post-translational modification, such as phosphorylation (Agarwala et al., 2000). Given the numerous reports on hypoxia-mediated up-regulation of NDRG1 in various cell lines and specifically in homocysteine-injured endothelium (Kokame et al., 1996), a role for this protein in protection from ischaemic cell damage was proposed. Ischaemic cellular stress leads to induction of HIF-1α (Semenza, 2000), a transcription factor that coordinates the up-regulation of numerous genes involved in cell survival mechanisms and angiogenesis (Sharkey et al., 2000; Semenza, 2002). Transcription of NDRG1 was shown to be dependent on HIF-1α activity (Salnikow et al., 2000b). Involvement of hypoxia-inducible genes in terminal differentiation/angiogenesis of the endometrium has been poorly explored to date. For instance, no study has yet documented in vivo the temporal regulation of HIF-1α in the differentiating human endometrium. However, the fact that expression of NDRG1 protein is maximal in the well-differentiated S-phase endometrium and reduced during the proliferative phase of this tissue is compatible with a growth inhibitory role observed in other systems (Kurdistani et al., 1998). It would also be of great interest to elucidate the transcriptional impact of estrogens and progesterone on the NDRG1 promoter and to investigate a potential synergistic effect of growth factors with sex steroid hormones.

In summary, this report represents a comprehensive study of the human NDRG1 temporal profile, both at mRNA and protein levels, in the endometrium throughout the menstrual cycle.

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References


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