Effects of urinary and recombinant gonadotrophins on gene expression profiles during the murine peri-implantation period

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BACKGROUND: Controlled ovarian stimulation (COS) with urinary gonadotrophins but not recombinant gonadotrophins, adversely affect the implantation process. In this study, we investigated the effects of urinary and recombinant gonadotrophins on gene expression profiles at implantation sites during the mouse peri-implantation period and the possible molecular mechanisms involved in the detrimental effects of urinary gonadotrophins using microarray technology. METHODS: Adult female CD1 mice were treated with (i) urinary human FSH (hFSH) and urinary HCG, (ii) recombinant hFSH and recombinant human LH or (iii) saline. Gene expression profiling with GeneChip mouse genome 430 2.0 arrays, containing 45,101 probe sets, was performed using implantation sites on embryonic day 5. Data were statistically analysed using Significance Analysis of Microarrays. Ten genes from the microarray analysis were selected for validation using quantitative RT–PCR (qRT–PCR). A parallel group of pregnant mice was allowed to give birth to study the effect of gonadotrophins on resorption. RESULTS: Urinary gonadotrophins differentially up-regulated the expression of 30 genes, increased resorption and reduced litter size, whereas recombinant gonadotrophins did not. Nine of the 10 genes were confirmed by qRT–PCR. CONCLUSIONS: Urinary gonadotrophins, but not recombinant gonadotrophins, exerted differential effects on gene expression during the murine peri-implantation period. These findings might contribute to improve protocols for COS, leading to higher successful pregnancy rates.

Key words: microarray/ovarian stimulation/peri-implantation period/recombinant gonadotrophins/urinary gonadotrophins

Introduction

Pregnancy outcome is dependent on the implantation of the blastocyst into the endometrial lining of the uterus which involves a series of co-ordinated and synchronized events in the development and maturation of the blastocyst and the development of the uterus into a receptive state (Psychoyos, 1973). Successful implantation is of clinical importance because only a very low percentage of embryos transferred after IVF lead to successful pregnancies. This is partly because of genetic abnormalities (Simpson and Liebaers, 1996) and mainly because of the non-receptivity of the uterus for reasons that are still unknown (Edwards, 1994).

Gonadotrophins are routinely used in human assisted reproduction techniques and in the generation of transgenic animals. Two types of gonadotrophins are used nowadays in controlled ovarian stimulation (COS): recombinant gonadotrophins produced by recombinant DNA technology and urinary gonadotrophins extracted from urine of post-menopausal women. In rodents, recombinant gonadotrophins do not exert any effect on the implantation process (Sibug et al., 2005), whereas urinary gonadotrophins exert deleterious effects such as increased frequency of chromosomal abnormalities (Maudlin and Fraser, 1977; Elbling and Colot, 1985; Luckett and Mukherjee, 1986), increased pre- and post-implantation mortality (Beaumont and Smith, 1975; Ertzeid and Storeng, 1992) and impaired implantation and prolonged gestation period (Ertzeid et al., 1993; Ertzeid and Storeng, 2001; Sibug et al., 2002, 2005). In humans, COS with urinary gonadotrophins resulted in gross alteration in the expression of endometrial genes in comparison with natural cycles (Horcajadas et al., 2005). In addition, COS with both recombinant FSH and urinary hCG resulted in reduced integrin expression in the endometrium (Thomas et al.,
2002). Integrins are expressed at the so-called ‘implantation window’ and are considered as useful markers for endometrial receptivity (Lessey et al., 1994). Hence, a reduction in their expression suggests a negative effect on pregnancy.

Several gene expression profiling studies on molecular factors involved in the implantation process have been performed. However, genome-wide analysis to assess and compare the effects of urinary and recombinant gonadotrophins during the mouse peri-implantation period has not yet been done. Understanding the molecular impact of gonadotrophins is of great importance because this will not only contribute to a better understanding of the implantation process but may also improve COS protocols. Therefore, this study was undertaken to assess and compare the effects of urinary and recombinant gonadotrophins on the expression profiles of genes during the mouse peri-implantation period using microarray technology. Total RNA was extracted from the implantation site on embryonic day 5 (ED5), and gene expression profiles were studied. Expression of selected genes was validated using quantitative RT–PCR (qRT–PCR). The data show that stimulation with urinary gonadotrophins altered the gene expression profiles during the peri-implantation period, whereas the recombinant gonadotrophins did not exert any significant change. To our knowledge, this is the first study that compared the effects of recombinant and urinary gonadotrophins on gene expression profiles of implantation sites during the peri-implantation period.

Materials and methods

Animals and experimental procedure

CD1 mice (8–10 weeks, Charles River, Germany) were housed five per cage upon arrival and allowed to acclimatize for 1 week. They had free access to food and water with a 12:12 dark : light cycle (lights on at 0700 h). All animal experiments were in accordance with the governmental guidelines for care and use of laboratory animals and approved by the Animal Care Committee of the University of Leiden.

The female mice (12 mice per group) were first injected (i.p.) with either of the following: (a) urinary human FSH (hFSH) (Metrodin; Serono, Coimbra, Switzerland), (b) recombinant hFSH (Gonal-F; Serono, Aubonne, Switzerland) or (c) saline at 12:00 pm, and 47 h later with urinary HCG (Pregnyl; Organon, Oss, The Netherlands), recombinant human LH (Lhadi; Laboratoires Serono, Aubonne, Switzerland) or saline, respectively. All gonadotrophins were given in a concentration of 5 IU in 0.1 ml saline. Based on this scheme, the following groups of mice were generated: (a) urinary gonadotrophin, (b) recombinant gonadotrophin and (c) saline (control). After the last injection, males were allowed to mate with the females (one male per two females). Successful mating was confirmed by the presence of a vaginal plug the following day (0800–0900 h). The day of vaginal plug detection was considered as ED0.

For microarray analysis, pregnant mice were injected with 0.1 ml 1% Chicago blue dye (Sigma-Aldrich, Steinheim, Germany) in the jugular vein under isoflurane anaesthesia to visualize the implantation sites (Psychoyos, 1973) on ED5. After allowing the dye to circulate for 10 min, the whole uterus with the embryos was dissected out and the animals were killed by decapitation. The uteri were cleared of fat tissue, immediately frozen in liquid nitrogen and stored at −80°C until further processing.

To assess pregnancy outcome, another set of saline-, urinary gonadotrophin- or recombinant gonadotrophin-treated pregnant mice (6–8 mice per group) were allowed to give birth and were sacrificed after delivery. The uteri were dissected out, and resorption (%) was determined from the difference between number of implantation sites and litter size.

Total RNA preparation and microarray hybridization

The site where the blastocyst was implanted (implantation site) was cut off on both sides from the adjoining intersites. Our previous studies showed that urinary gonadotrophins can delay implantation and decrease vascular permeability, and these are reflected by a smaller size and absence or less intensity of dye staining (Sibug et al., 2002, 2005). The expression profiles of genes exhibit a temporal pattern and some genes become active only at certain critical periods (Hamatani et al., 2004). To exclude these confounding effects of different developmental stages, we used only implantation sites with comparable size and dye staining. The position of implantation sites did not show a distribution pattern. Hence, they were randomly collected from the different parts of the uterus as long as they met the mentioned criteria.

Total RNA (from three to four implantation sites per mouse, pooled) was extracted using TRIzol reagent (Invitrogen, Carlsbad, NM, USA) and used as a template for complementary DNA (cDNA) synthesis and biotinylated antisense cRNA preparation. The quality and amount of total RNA were characterized on nanochips using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The synthesis of cDNA and cRNA, labelling, hybridization and scanning of the samples were performed as described by Affymetrix (www.affymetrix.com). GeneChip mouse genome 430 2.0 arrays, each containing 45 101 probe sets, (Affymetrix; Santa Clara, CA, USA) were used to hybridize the labelled cRNA. Four gene chips (n = 4 animals) per group were used giving rise to a total of 12 gene chips (n = 12 animals) for three groups. Each gene chip contained a sample from three to four pooled implantation sites from one mouse. Hybridization and scanning of the probe arrays were done at the Leiden Genome Technology Center (for details, see www.lgtc.nl) with Fluidics Station 450 and Gene Chip Scanner 3000.

Data analysis for microarray

Microarray Analysis Suite 5.0 (MAS5.0; Affymetrix) was used to estimate signal intensities plus signal reliabilities and to normalize the array signals by total intensity normalization (Quackenbush, 2002). Briefly, a given transcript is represented by a probe set consisting of 11 perfect/mismatch probe pairs. The software determines the degree of detection (detection call) based on the signal intensity, background noise and variation of the expression signals within the probe pairs. Present, marginal or absent calls have \( P \leq 0.04, 0.04–0.06 \) or \( >0.06 \), respectively.

The reliability of the data was ensured by excluding probe sets with absent call on all 12 chips. Then the \( P \)-values of the detection calls generated by Affymetrix were used to calculate the mean \( P \)-value of each probe set per experimental group (\( n = 4 \)). Probe sets with a mean \( P \geq 0.06 \) were called group Absent (gA), \( P = 0.04–0.06 \) were called group Marginal (gM) and \( P \leq 0.04 \) were called group Present (gP). Probe sets that were gA in all groups (3gA), gM in all groups (3gM), gA in two groups and gM in one group (2gA + 1gM) and gA in one group and gM in the other two groups (1gA + 2gM) were also excluded. Consequently, only probe sets with at least 1gP were considered reliable for statistical analysis. Significance Analysis of Microarrays (SAM) (Tusher et al., 2001) was used to identify responsive genes. SAM is a distribution-free test, which allows the user to control the false discovery rate (FDR), i.e. the relative number of false positives generated. An FDR of 63% and a fold change of \( \geq 1.5 \) were set to generate the number of significant genes. An FDR of 63% was chosen because lower FDRs resulted in a very limited number of genes. A fold change of \( \geq 1.5 \) was chosen because pilot studies showed...
that fold changes <1.5 were difficult to validate with qRT–PCR. Gene Ontology Biological Process classifications were obtained using the NetAffx Analysis Center (www.affymetrix.com), allowing genes with a similar biological process to be grouped together.

qRT–PCR
The specificity of the genes obtained from the microarray analysis was tested by qRT–PCR, using the same total RNA samples used for array hybridization. For each gene, four mice/group were used and run in duplicates. Total RNA was DNase-treated followed by cDNA synthesis. Briefly, the following were added together in a total volume of 20 μl: 10 μl of total RNA (1 μg), 1.5 μl of oligo dT (0.5–1 μg/μl; Invitrogen), 1 μl (10 mM) dNTP (Invitrogen), 2 μl 10× M-Moloney Murine Leukemia Virus (MuLV) reaction buffer (Finnzymes; Espoo, Finland), 1 μl RNaseOUT (40 U/μl, Invitrogen) and 4.5 μl of M-MuLV Reverse Transcriptase (200 U/μl; Finnzymes). For the negative controls, RNase-free water was added instead of the Reverse Transcriptase enzyme. The mixture was incubated for 1 h at 42°C and then the enzyme inactivated at 70°C for 15 min.

Primers were generated using the Primer3 Input program (primer3_www.cgi v 0.2). Primers were designed in the same region as the probe set on the gene chip. The selected primers were controlled for specificity using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The primers used are summarized in Table I. Serial dilutions of a known amount of cDNA sample were used for the standard curves of the genes. The housekeeping gene β-actin was used for normalization because it was not affected by the different gonadotrophin treatments. The LightCycler kit (LightCycler® FastStart DNA Master+SYBR Green I; Roche, Mannheim, Germany) was used to prepare the qRT–PCR mix. In each PCR, 5 μl of cDNA, 2 μl of forward primer (10 μM), 2 μl of reverse primer (10 μM) and 4 μl of the LightCycler kit mix were added in a 20-μl reaction mixture. The samples, in duplicates, were amplified with an annealing temperature of 60°C using the LightCycler 2.0 (Roche). The protocol used to amplify all the genes was 45 cycles. However, the threshold cycle (Ct) values used to compute for the concentration of the different genes varied and, in general, were within the range of 19–28.

The concentrations and melting temperatures of the different samples for each gene were calculated using the LightCycler Software 4 (Roche). For every sample, the ratio of expression was determined by dividing the concentration of the target gene by that of β-actin. The computed ratios were then statistically tested with one-way analysis of variance and the post hoc least significant difference test. Significance was accepted with P ≤ 0.05.

Table I. List of primers used for quantitative RT–PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
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<tr>
<td>Efemp</td>
<td>TTGTCGCTTCCTGCAAATGG</td>
<td>GCATGCTTAAGATGGCAATTC</td>
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</tr>
<tr>
<td>Spr2f</td>
<td>CTTGGTGCCACCAAAAG</td>
<td>GCATTCTCTGCTGAAAGG</td>
<td>128</td>
</tr>
<tr>
<td>Kldh4c</td>
<td>GAAAACCTTGGGAGGATGG</td>
<td>GGACAGTGACTCTGCAATTC</td>
<td>191</td>
</tr>
<tr>
<td>H2-K1</td>
<td>CATGCCATGTCGCACATAG</td>
<td>ATTCACGCTCAGAAAGG</td>
<td>118</td>
</tr>
<tr>
<td>Emb</td>
<td>TGCATGGGCCACTACATT</td>
<td>CATAAAGGCCACCAAGA</td>
<td>229</td>
</tr>
<tr>
<td>Ifi3</td>
<td>ATGGGCTACTGAGGACATCT</td>
<td>CTTGTTCATGAGGACATCT</td>
<td>111</td>
</tr>
<tr>
<td>Oasl</td>
<td>CAAACATCTCTGTGAGCTG</td>
<td>GGACATGGCAAGTAAGTGC</td>
<td>193</td>
</tr>
<tr>
<td>Fli2</td>
<td>ATTTTCAGGATGACATATG</td>
<td>CCCATTGCGGAGAAGAAT</td>
<td>223</td>
</tr>
<tr>
<td>B2m</td>
<td>CTGGTCTTTCCTGTTTGT</td>
<td>GTATTGGCTGGCTCCCCATT</td>
<td>109</td>
</tr>
<tr>
<td>Gdf10</td>
<td>AAACCTCAGGGTCTCATC</td>
<td>GACACCCACACCTAAACTG</td>
<td>219</td>
</tr>
</tbody>
</table>

B2m, beta-2 microglobulin; Efemp1, epidermal growth factor-containing fibulin-like extracellular matrix protein 1; Emb, embigin; Fli2, fibrinogen-like protein 2; Gdf10, growth differentiation factor 10; H2-K1, histocompatibility 2, K1, K region; Ifi3, interferon-induced protein with tetratricopeptide repeats 3; Kldh4c, kelch domain containing 4; Oasl, 2′–5′ oligoadenylate synthetase-like 2; Spr2f, small proline-rich protein 2F.

Results

Resorption
The degree of decidualization in the primary and secondary decidual zone and the developmental stage of the embryos were comparable in all the three groups (Figure 1).

The frequency of embryo loss was significantly higher in the urinary gonadotrophin-treated group (n = 7) in comparison with the saline group (n = 11) (32.3 ± 9.9 versus 5.2 ± 2.4%, respectively; Figure 2). No significant difference was observed between the recombinant gonadotrophin-treated group (n = 6) and the saline group (n = 11) (8.3 ± 2.3 versus 5.2 ± 2.4%, respectively). Litter size was significantly lower in urinary gonadotrophin-treated group than in saline-treated group but not in the recombinant-treated group (5.4 ± 1.3, 12.2 ± 1.0 and 12.0 ± 0.7, respectively).

Microarray expression profiling
Filtering out all probe sets with absent calls on all 12 chips resulted in 29 373 of 45 101 probe sets. Subsequent exclusion of probe sets containing 3gM, 3gA, 2gM + 1gA and 1gM + 2gA led to 20 966 genes, which comprise only 46% of all probe sets present in the chip and were used for further analysis. SAM analysis of the microarray data showed that a set of
genes was differentially expressed between the urinary gonadotrophin and the control group during the peri-implantation period. At an FDR of 63%, stimulation with urinary gonadotrophins significantly up-regulated the expression of 30 genes (Table II) and did not significantly down-regulate any gene. The recombinant gonadotrophins did not alter the gene expression profiles at all.

Based on gene ontology classification, the majority of the differentially expressed genes have biological functions related to the implantation process such as immunoregulation, cell adhesion, transcription, intracellular signalling, receptor activity and metabolism (Table II). However, there are a number of genes whose biological functions and relevance to the implantation are still unknown.

**qRT–PCR**

The following 10 genes were selected based on fold change and/or putative function in implantation process and tested using qRT–PCR for confirmation: epidermal growth factor-containing fibrinulin-like extracellular matrix protein 1 (*Efemp1*), small proline-rich protein 2F (*Sprr2f*), kelch domain containing 4 (*Klhdc4*), histocompatibility 2, K1, K region (*H2-K1*), embigin (*Emb*), interferon-induced protein with tetrascaropeptide repeats 3 (*Ifit3*), 2′-5′ oligoadenylate synthetase-like 2 (*Oas1l*), fibrinogen-like protein 2 (*Fgl2*), β-2 microglobulin (*B2m*) and growth differentiation factor 10 (*Gdf10*). Except for *Klhdc4*, all the rest showed enhanced gene expression upon treatment with urinary gonadotrophins, confirming the microarray dataset. Note that the fold changes in the expression levels were higher in qRT–PCR than in the microarray analysis (Figure 3).

**Discussion**

The implantation process is a complex process orchestrated and synchronized by the development of the embryo and preparation for receptivity of the uterus. Different sets of genes undergo activation/deactivation at different stages of development to pave the way for a successful implantation of the blastocyst in the uterine lining. In this study, stimulation with urinary gonadotrophins, but not recombinant gonadotrophins, exerted differential effects on gene expression during the peri-implantation period. These gene expression effects might be related to negative effects on implantation because urinary gonadotrophins compared with saline treatment also increased resorption resulting in a small litter size.

These results are in accordance with our previous findings (Sibug et al., 2002, 2005) in which we demonstrated that urinary gonadotrophins led to a delayed implantation, reduced vascular permeability, reduced expression of the vascular endothelial growth factor system, retarded growth and prolonged gestational period. In contrast, the recombinant gonadotrophins did not exert any effect on these parameters. The qRT–PCR results clearly confirmed the microarray data with nine of the 10 genes selected for independent testing showing a change in expression with both methods. The validation of nine of the 10 genes is higher than that one would expect from an FDR of 63%. In general, the fold changes observed in the qRT–PCR are higher than those observed in the microarray data, a phenomenon that is often observed when comparing array hybridization and qRT–PCR results (Rajeevan et al., 2001; Bangur et al., 2002). Two of the 10 genes showed more than 2-fold difference (5.1× for *Efemp1* and 3.2× for H2K-1) but for the majority the qRT–PCR results and microarray data corresponded well. This difference in fold changes between the microarray and qRT–PCR data is not unique in our hands. In fact, even larger differences, ranging from 9× to 21×, were reported by Rajeevan et al. (2001) and Bangur et al. (2002) and reflect the difference when using different techniques to assess messenger RNA expression.

Gene expression profiling studies in the developing oocyte and uterus before and during the peri-implantation period show that expression profiles of genes are dynamic and exhibit a spatio-temporal pattern (Yoshioka et al., 2000; Hamatani et al., 2004; Wang et al., 2004). Several genes are turned on and off at several stages of the developing oocyte until the activated blastocyst by waves of activation and deactivation. Subsequently, genes become active only at certain critical periods (Hamatani et al., 2004). Furthermore, it is possible that the activated and deactivated genes may have downstream effects on other genes. It is, therefore, possible that the changes in the gene expression profiles with urinary gonadotrophins on ED5 might be the result of a series of changes that took place in the developing oocyte and uterus before and until this time point.

Independent effects on the developing oocyte and uterus can also occur. It has been shown, for example, that unfertilized oocytes from ovulated female hamsters had an aberrant microfilament formation in comparison with the non-treated (Lee et al., 2005). In vitro experiments in rodents showed that the quality of developing oocytes/blastocysts is adversely affected by urinary gonadotrophins (Elbling and Colot, 1985; Edgar et al., 1987; Ertzeid and Storeng, 1992; Van der Auwera and D’Hooghe, 2001). Embryo transfer experiments in mice showed that prenatal loss of embryos and abnormal embryonic development after stimulation with urinary gonadotrophins are
Gonadotrophins and gene expression at implantation sites

predominantly induced by effects of the hormone treatment on the maternal uterine environment (Elmazar et al., 1989; Spielmann and Vogel, 1993). Clinical data on COS also indicate that gonadotrophins might exert their effects only in the uterus. Birthweight of singletons conceived by implanting a cryopreserved embryo is significantly higher than birthweight after a fresh embryo transfer (Wennerholm, 2000; Wang et al., 2005).

Because cryopreserved embryo transfer occurs predominantly in a natural cycle, while fresh embryo transfer in a hormonally induced state, one may hypothesize that COS might only influence uterine receptivity. Regardless of frozen or fresh embryos, it appeared that uterine environments were affected by the urinary gonadotrophin treatment, resulting in the outcome of pregnancy success.

The increased expression of Fgl2 (synonym prothrombinase) might be involved in the increased resorption observed in the mice treated with urinary gonadotrophins. Fgl2 is an enzyme which converts prothrombin to thrombin and has been shown to be required for normal reproduction in mice (Clark et al., 2004). Interestingly, it has been correlated with spontaneous abortion in rodents (Clark et al., 1999a,b) and recurrent spontaneous abortions in humans (Knackstedt et al., 2001).

**Table II.** List of significantly up-regulated genes with urinary gonadotrophins based on microarray analysis

<table>
<thead>
<tr>
<th>GenBank Accession number</th>
<th>Gene title</th>
<th>Gene symbol</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC023060.1</td>
<td>Epidermal growth factor-containing fibulin-like extracellular matrix protein 1</td>
<td>Efemp1</td>
<td>1.6</td>
</tr>
<tr>
<td>NM_010330.1</td>
<td>Embigin</td>
<td>Emb</td>
<td>1.6</td>
</tr>
<tr>
<td>NM_019759.1</td>
<td>Dermatopontin</td>
<td>Dpt</td>
<td>1.7</td>
</tr>
<tr>
<td>S70184.1</td>
<td>Histocompatibility 2, K1, K region</td>
<td>H2-K</td>
<td>1.8</td>
</tr>
<tr>
<td>NM_010501.1</td>
<td>Interferon-induced protein with tetratricopeptide 3</td>
<td>Ifit3</td>
<td>3.9</td>
</tr>
<tr>
<td>AF119253.1</td>
<td>Histocompatibility 2, class II antigen A, alpha</td>
<td>H2-Aa</td>
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<tr>
<td>BQ033138</td>
<td>2′-5′ oligoadenylate synthetase-like 2</td>
<td>Oas2l</td>
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<tr>
<td>BM241485</td>
<td>Macrophage activation 2 like</td>
<td>Mps2l</td>
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<td>AI099111</td>
<td>Beta-2 microglobulin</td>
<td>B2m</td>
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<td>LA2114.1</td>
<td>Growth differentiation factor 10</td>
<td>Gdf10</td>
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<td>X17502.1</td>
<td>Branched chain aminotransferase 1, cytosolic</td>
<td>Bcat1</td>
<td>1.7</td>
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<tr>
<td>BC013464.1</td>
<td>Ketohexokinase</td>
<td>Khk</td>
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<tr>
<td>AK011543.1</td>
<td>General transcription factor II E, Polypeptide 1 (alpha subunit)</td>
<td>Gtf2e1</td>
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<tr>
<td>NM_023292.1</td>
<td>Pseudouridine synthase 3</td>
<td>Pus3</td>
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<tr>
<td>NM_053117.1</td>
<td>par-6 partitioning defective 6 homolog gamma (Caenorhabditis elegans)</td>
<td>Pard6g</td>
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<td>BB828559</td>
<td>Mitogen-activated protein kinase kinase 5</td>
<td>Map2k5</td>
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<td>BB236260</td>
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<td>Gpr172b</td>
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<tr>
<td>NM_011150.1</td>
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<td>BC024542.1</td>
<td>Oncoprotein-induced transcript 1</td>
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<td>NM_023197.1</td>
<td>RIKEN cDNA 2310008H09 gene</td>
<td>2310008H09Rik</td>
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*Genes tested for validation with quantitative RT–PCR.

**Figure 3.** Comparison of fold changes of the differentially up-regulated genes obtained from microarray analysis and quantitative RT–PCR (qRT–PCR).
These abortive effects might be because of immunoregulatory processes taking place during pregnancy and might be explained by the Th1/Th2 paradigm. According to this paradigm, the balance of pro-inflammatory T helper (Th1) cytokines and anti-inflammatory Th2 cytokines and the local dominance of the Th2 cytokines, which protects the semi-allogenic embryo against both adaptive cell-mediated immune reaction, non-specific innate inflammatory and phagocytic responses, are critical for a successful pregnancy (Wegmann et al., 1993). Th1 cytokines are known to up-regulate FgI2 and via thrombin leads to activation of polymorphonuclear leukocytes. These, in turn, trigger inflammation and fibrin deposition leading to embryonic death in mice (Clark et al., 1998, 2001). The presence of FgI2 in the decidua and trophoblasts of aborted but not in control tissue (Clark et al., 1999a) suggests that even normal looking implantation sites treated with urinary gonadotrophins can experience higher gene expression resulting in resorption. The validity of the Th1/Th2 paradigm to solely explain pregnancy has been challenged (Chaoiat et al., 2004) but nevertheless, its involvement in embryo loss observed in this study cannot be excluded. On the contrary, an apoptotic effect of FgI2 might be involved. FgI2 also has a direct apoptosis-inducing effect on cells, and apoptosis of trophoblast cells is a feature of spontaneous abortion (Knackstedt et al., 2001).

Implantation of the blastocyst involves adhesion, attachment and invasion of the uterine wall. Adhesion of the blastocyst on the uterine lining requires acquisition of attachment competence and functional expression of complementary adhesion-promoting molecules at the cell surface of the blastocyst and uterine lining (Carson et al., 1994). Blastocyst attachment and invasion of the uterine lining are accompanied by extensive degradation and remodelling of the extracellular matrix (ECM). The latter is regulated by a balance of metalloproteinases (MMP) and their inhibitors (Alexander et al., 1996). The cell adhesion molecules, Efemp1 and Emb, are among the genes which were significantly up-regulated by urinary gonadotrophins on ED5. Efemp1 might have affected the implantation process via strong binding with tissue inhibitor of metalloproteinases-3 (Timp-3) (Klenotic et al., 2004). Timp-3 suppresses ECM degradation (Klenotic et al., 2004) and acts as a major MMP inhibitor in the murine uterus by restricting invasion to the implantation site (Leco et al., 1996). Hence, although indirect, it is not preposterous to assume that the urinary gonadotrophin-induced Efemp1 expression might have hampered the implantation process by inhibiting ECM degradation via Timp-3. Emb, on the contrary, is a glycoprotein that promotes integrin-mediated cell substratum adhesion and regulates cell/ECM interaction during development. Its presence in the uterus of non-treated pregnant mice in considerable amounts (Huang et al., 1990) implies a significant role in the implantation process. As one would likely assume that its increased expression would have beneficial consequences, we cannot offer a possible mechanism on how it can negatively affect the implantation process.

The implantation process elicits a pro-inflammatory reaction, and the mechanism whereby the semi-allograft embryo escapes paternal immune response during pregnancy is still unknown (Billington, 1992). Reese et al. (2001) suggested that, during blastocyst implantation, a large number of immune-related genes are absent, and those present have reduced expression and remain inactive with the onset of implantation. The enhanced expression of the immune response genes H2-K1, Ifi3, B2m and Gdf10 is in accordance with this suggestion and might have contributed to the increased resorptions observed in the urinary gonadotrophin-treated animals. It should be noted that the inflammatory reaction might not be elicited solely by the semi-allogenic embryo though. The urinary gonadotrophins are not as pure as the recombinant gonadotrophins, and therefore, the presence of degradation products cannot be ruled out. This suggestion is supported by the finding that degradation products are present in urinary hCG (Wehmann and Nisula, 1980).

We do not know the significance of the alteration in expression of the gene Sprr2f1 in the present study. Sprr2f1 is a structural component of the cornified cell envelope of stratified squamous epithelia. Its distinct expression in the uterus (Song et al., 1999; Reese et al., 2001) is difficult to explain because the uterus does not contain stratified squamous epithelia. On the contrary, Sprr2f1 is not the only differentially expressed gene whose function is in question in the present study. As shown in Table II, there are a number of altered genes whose role in the implantation process remains to be discovered.

The use of CG instead of LH in the uterine group might have also contributed to the differential effects observed in this study. CG is normally used to mimic the effects of pituitary LH because they have a high degree of homology and bind to the same membrane receptors. However, their molecular composition differs (Wehmann and Nisula, 1980; Stokman et al., 1993), indicating that their biological actions may not be identical (Niswender et al., 1985). Data showing that treatment with LH produces better quality embryos with a higher implantation rate than CG in New Zealand rabbits (Peinado et al., 1995), and a higher number of implantation sites with greater vascular permeability in mice (Sibug et al., 2005) support this assumption.

The urinary gonadotrophin-treated uteri/embryos evaluated in the present study appeared normal and will most likely have survived until birth and beyond despite the hormonal treatment. However, there was an up-regulation of 30 genes in the urinary gonadotrophin-treated implantation sites despite a similar degree of decidualization, developmental stage and vascular permeability in comparison with the controls. This indicates that even normal looking uteri/embryos were facing the negative effects of urinary gonadotrophins.

In summary, urinary gonadotrophins, but not recombinant gonadotrophins, increased resorption and altered gene expression profiles during the peri-implantation period. The altered expressions of these genes might be some of the underlying factors in increased resorption of embryos in females treated with urinary gonadotrophins. Although caution must be taken in interpreting these results and extrapolating them to the human situation, we believe that these findings warrant careful consideration because COS with urinary gonadotrophins in humans resulted in a dramatic disturbance in the expression of endometrial genes (Horcajadas et al., 2005). Moreover, these
results might contribute to the improvement of COS leading to successful pregnancy. Further studies are necessary to determine whether the effects of urinary gonadotrophins on gene expression profiles during the peri-implantation period observed in this study exert long-term consequences in the offspring.

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References

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