Effect of low-dose mifepristone administration on day 2 after ovulation on transcript profiles in implantation-stage endometrium of rhesus monkeys

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Abstract

Progesterone is essential for endometrial receptivity in primates. In studies previously performed using global gene profiling based on microarray technology, attempts have been made to identify changes in gene expression between early luteal-phase and mid-luteal-phase endometria. However, the issue of the putative impact of preimplantation embryo-derived signal in the process of endometrial receptivity was missing in the previous studies. In the present study, an attempt has been made to delineate the transcripts profile in implantation-stage endometrium under combinatorial regulation of progesterone and embryo-derived signal in the rhesus monkey. To this effect, we have compared transcript profiles for 409 known genes between control receptive stage ($n=13$), and mifepristone-induced desynchronized and non-receptive stage ($n=12$) monkey endometrial samples collected on days 4 ($n=12$) and 6 ($n=13$) after ovulation from mated, potential conception cycles, using cDNA arrays containing sequence-verified clones. Statistical analysis of correlation of estimated transcript abundance between arrays and qRT-PCR for nine selected gene products yielded significant ($P<0.05$) concordance. Of 409 genes, a total of 40 gene transcripts were seen to be affected, nine gene transcripts in endometrial samples were found to progressively increase between days 4 and 6 following mifepristone treatment, while an additional five genes showed differential expression profile depending on the day after treatment. Additionally, different sets of 12 and 14 gene products showed changes in days 4 and 6 post-ovulation samples respectively. A new cohort of 28 gene products in implantation-stage endometrium was seen to be affected by luteal-phase mifepristone.

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Introduction

Typically, the mid-luteal phase of a normal mated ovulatory cycle provides the implantation window in the monkey and the human (Hendrickx & Enders 1980, Edwards 1995). There are a few reports wherein the transcript profile of mid-luteal-stage endometrium has been compared with that of early luteal-stage endometrium to delineate the role of progesterone in establishing endometrial receptivity for embryo implantation (Carson *et al.* 2002, Kao *et al.* 2002, Borthwick *et al.* 2003, Riesewijk *et al.* 2003, Horcajadas *et al.* 2004). However, this model has a limitation; substantial biochemical and morphological evidence suggests that mid-luteal-phase endometrium exhibits phenotypically differential characteristics only in the presence of viable, synchronous embryo (Ghosh *et al.* 1993, Ghosh & Sengupta 1998, 2004, Simon *et al.* 2000, Simon & Dominguez 2004). Moreover, mifepristone administered at a relatively small dose (2 mg per kg body weight) in the early luteal stage renders endometrium phenotypically

hostile during the implantation window resulting in implantation failure without any discernible change in serum concentrations of oestradiol and progesterone (Gemzell-Danielson *et al.* 1993, Ghosh & Sengupta 1993). In the present study, we performed comparative analysis of selected transcript profiles between endometrial samples collected from fecund cycles of rhesus monkeys on days 4 and 6 after ovulation with and without mifepristone (2 mg per kg body weight) treatment on day 2 after ovulation. We report here for the first time that a set of genomic expression showed an overt differential regulation in implantation-stage endometrium when it had been subjected to early luteal-phase treatment with high-affinity antiprogesterin like mifepristone.

Results

Out of 36 animals who were assigned arbitrarily into control and treatment subgroups under both groups, two failed to show any clear indication of ovulation and

six failed to yield any embryo on uterine flushing. Thus, preimplantation embryos between morula and blastocyst stages from the uteri of 28 animals could be retrieved and endometrial samples from these animals were collected for the present study. However, three samples were discarded because of the unacceptable quality of extracted RNA on quality assessment (Table 1). The details of the recovered embryos have been given elsewhere (Ghosh *et al.* 1997, 2000, Lalitkumar *et al.* 2005a).

Figure 1 shows the results of typical analysis of concordance of normalized expression profiles between control receptive endometrium and mifepristone-treated non-receptive endometrium on days 4 (Fig. 1A) and 6 (Fig. 1B) after ovulation. Overall, it reveals that the ranges of average expressions at the transcription level were relatively higher on day 6 after ovulation compared with that on day 4 after ovulation in both groups, presumably concurring with secretory-stage maturation of endometrium.

Statistical analysis of normalized, log-transformed, filtered cDNA array data of 409 gene products using conventional statistical tools identified that a total of 26 genes (20 increased and 6 decreased) on day 4, and 28 genes (25 increased and 3 decreased) on day 6 after ovulation displayed statistically significant differential expression in endometrium as a result of mifepristone treatment on day 2 after ovulation in proven fertility cycles. As shown in Table 2, out of 409 genes relative abundance of transcripts of nine gene products (matrix metalloproteinase 9 (MMP9), STAT3, kinase domain receptor (KDR), tasin, RSP9, JUNB, leptin receptor, transforming growth factor β -1 (TGFB1) and RSP7) was higher in endometrial samples on both days 4 and 6 after ovulation following mifepristone treatment as compared with control samples and these showed a progressive increase between days 4 and 6 after ovulation in the mifepristone-treated group. A set of another 12 gene products showed changes only in day 4 post-ovulation samples, ten of them were increased and two showed decreased expression following mifepristone treatment (Table 2). Similarly, a different set of 14 gene products showed changes only in day 6 samples, 12 of them were

increased and 2 were decreased following mifepristone treatment (Table 2). An additional five gene products were seen to express differentially depending on the day after treatment: transcript abundance for colony stimulating factor 1 (CSF1), RASA1, uteroferrin and uteroglobin were lower on day 4 after ovulation but reversed to become higher on day 6 after ovulation following mifepristone treatment while a reciprocal relationship was seen for *enigma* (Table 2). Statistical analysis based on gene expression statistical system software for repeated measures identified all 40 gene products to show differential changes, except three gene products (MMP9, CSF1 and RASA1) in samples collected from day 4 after ovulation (Table 2). Real-time quantitative RT-PCR verification for nine products using probes and primers as given in Table 3 confirmed the reliability of the observed changes in array analysis (Table 4).

Immunohistochemical examination for the protein products of JAK1 and activated STAT3 in endometrial samples collected on days 4 and 6 after fertilization with and without mifepristone treatment (Table 5, Figs 2 and 3) revealed that the immunopositive signals for JAK1 and pSTAT3 were consistently and markedly higher in the epithelial compartment ($P < 0.01$) compared with the stromal and vascular compartments on days 4 and 6 after ovulation, and mifepristone administration on day 2 after ovulation yielded a trend of reversing the profile resulting in lowered trend of score in the epithelial compartment and increased trend of score in stromal and vascular compartments.

Discussion

In the present study, we compared transcript profiles for known 409 genes between 'receptive', and mifepristone-induced desynchronized 'non-receptive'-stage monkey endometrial samples from mated, potential conception cycles, using cDNA arrays containing sequence-verified clones already known to be important in endometrial function. Out of 409 genes analysed following normalization, a total of 40 (10%) gene transcripts were seen to be affected, 9 (~2%) gene transcripts in endometrial samples were found to

Table 1 Details of treatment groups.

Group	Subgroup (n) ^a	Treatment description			Number of usable samples obtained ^b
		Agent injected	Post-ovulation day of		
			Injection	Tissue collection	
1	a (6)	Vehicle ^c	2	4	6
	b (8)	Mifepristone ^d	2	4	6
2	a (7)	Vehicle ^c	2	6	7
	b (7)	Mifepristone ^d	2	6	6

^a*n* values show numbers of animals that yielded preimplantation-stage embryos in the respective subgroups. ^bBased on RNA integrity on electrophoresis, yield and RIN score as described in Materials and Methods. Animals that failed to yield preimplantation stage embryos (*n*=6) and failed to ovulate (*n*=2) are not shown. ^c2 ml, benzyl benzoate: olive oil (1:4), s.c. ^d2 mg/kg body weight in 2 ml vehicle, s.c.

Figure 1 Scattergrams showing microarray analyses comparing the mRNA expression (in logarithmic scale) profiles between control receptive (X-axis) and mifepristone (Y-axis)-treated endometrial samples collected on days 4 (A) and 6 (B) after ovulation. Each point represents normalized mean signal intensities of candidate probes following hybridization.

progressively increase between days 4 and 6 following mifepristone treatment, while an additional 5 (~1%) genes showed differential expression profiles depending on the day after treatment. A different set of 12 (3%) gene products showed changes in day 4 sample only, 10 of them were increased following mifepristone treatment, while another set of 14 (~4%) gene products showed changes only in day 6 samples, 12 of them were increased following mifepristone treatment. We have validated our cDNA array-based data using TaqMan qPCR for nine gene products with observed significant concordance between the two methods. Collectively, it appears that 31 out of 54 gene products with differential expression levels showed changes within fivefold, while residual 23 gene products showed more than fivefold changes in post-ovulation endometrium; however, only four specific gene products showed overlapping between days for the similar levels of fold changes in endometrium following mifepristone treatment. However, the physiological significance of fold changes,

as pointed out by Sherwin *et al.* (2006), remains conjectural. In this way, the systems biology approach in attaching informational value to relative abundance of signals irrespective and independent of its most logical output (Shannon 1949, Laughlin 2001) appears to be an attractive proposition in understanding the molecular biology of endometrial receptivity.

Table 6 gives a list of common gene products that appeared to be regulated by progesterone in previous reports and those reported to display differential abundance in the present study. Now, we report for the first time that a new cohort of 28 gene products were affected in implantation-stage endometrium, following early luteal-phase administration of mifepristone in the present study, and those that were not reported to be affected by progesterone in earlier array-based studies using the model of comparative study between pre-receptive and receptive stage endometria (Carson *et al.* 2002, Kao *et al.* 2002, Borthwick *et al.* 2003, Riesewijk *et al.* 2003, Giudice 2004, Horcajadas *et al.* 2004, Mirkin *et al.* 2005, Talbi *et al.* 2006, Sherwin *et al.* 2007). Out of nine gene products that were subjected to real-time RT-PCR quantification, seven (CD44, CSF1, distal less homeobox 4 (DLX4), enigma, KDR, leptin receptor, macrophage migration inhibitory factor (MIF)) are reported here as novel candidates, while two (mucin 1 (MUC1) and uteroglobin) have been reported earlier (Table 6). While previous reports indicate that *wnt*-associated pathways are involved in progesterone action on endometrial differentiation (Talbi *et al.* 2006), changes in transcript profiles of homeobox genes like DLX4 and enigma, as well as, of transcription and translation-related gene products like JUNB, JUN, elongation factor 1- α (ELF1), ribosomal proteins in implantation stage endometrium following mifepristone treatment have not been reported earlier.

Higher number of copies for transcripts of cyclooxygenase and receptor for prostaglandin F₂ on day 6 after ovulation were seen in mifepristone-treated groups. Also, higher transcript levels for TGF β 1 and MMPs on both days 4 and 6, and tissue necrosis factor (TNF) on day 6 after ovulation were seen in the mifepristone-treated group. Relative abundance of transcripts for prolactin receptor and mucin became lower in mifepristone-treated endometrium on days 4 and 6 respectively after ovulation, compared with control endometrium. These factors are known to be regulated in endometrium by progesterone (Salamonsen & Woolley 1996, Aplin *et al.* 1998, Ghosh *et al.* 1998, Nayak *et al.* 1998, Kim *et al.* 1999, Marions & Gemzell-Danielsson 1999, Tseng & Mazella 1999, Milne & Jobbour 2003, Lalitkumar *et al.* 2005a, 2005b, Stavreus-Evers *et al.* 2005, Critchley *et al.* 2006, Goff *et al.* 2006, Slayden & Brenner 2006, Makino *et al.* 2007). It may be conjectured that significant changes in the expression of these factors are involved in endometrial hostility in mifepristone-treated endometrium.

Table 2 Mean fold change in implantation-stage endometrial gene expression in response to mifepristone (2 mg/kg body weight, s.c.) treatment on day 2 after ovulation.

Category (Gene name)	Gene ID	Fold change	
		Day 4PO	Day 6PO
Increased on both days			
Matrix metalloproteinase 9	J05070	2.1 ^{a,*}	3.7*
STAT3	L29277	2.7*	3.9*
Kinase domain receptor	NM_002253	2.9*	11.0 [†]
Tastin	U04810	4.1*	5.6*
Ribosomal protein S9	U14971	4.1*	60.5 [†]
<i>JunB</i>	M29039	4.0*	62.5 [†]
Leptin receptor	U43168	15.5 [†]	27.5 [†]
<i>TGFB1</i>	X02812	74.5 [†]	94.2 [‡]
Ribosomal protein S7	BC061901	76.6 [‡]	342.0 [§]
Decreased on both days			
None			
Increased only on day 4			
<i>wif</i>	AY358344	2.6*	—
<i>Follistatin-like 1</i>	M19481	2.8*	—
Tissue factor (thromboplastin)	NM_001993	2.9*	—
Bystin	L36720	3.2*	—
3 β hydroxysteroid dehydrogenase	M27137	4.7*	—
Integrin α -6	X53586	4.8*	—
STAT-PIAS γ	AF077952	6.3*	—
Keratan sulphotransferase1	AF219990	16.4 [†]	—
<i>α-1 anti-trypsin</i>	K01396	32.6 [†]	—
<i>Matrix metalloproteinase 3</i>	X05232	42.9*	—
Decreased only on day 4			
<i>Prolactin receptor</i>	S78505	4.4*	—
ErbB3	NM_00198	7.0*	—
Increased only on day 6			
Cyclooxygenase (COX) 1	HSU63846	—	2.1*
Elongation factor 1- α	AF458103	—	2.2*
IL6	X04602	—	2.2*
<i>JAK1</i>	M35203	—	2.7*
Distal less homeobox 4	NM_138281	—	2.9*
Tissue necrosis factor- α	X02910	—	3.9*
JUN	NM_002228	—	4.0*
<i>PGF receptor</i>	L24470	—	4.8*
Macrophage inhibitory factor	NM_002415	—	6.9*
CD44	M59040	—	7.0*
Thioredoxin	J04026	—	7.2 [†]
Matrix metalloproteinase 10	X07820	—	92.3 [‡]
Decreased only on day 6			
17, 20 desmolase	U14953	—	2.0*
Mucin 1	NM_182741	—	2.1*
Differential responses between days 4 and 6			
CSF1	NM_000757	2.3 ^{a,*} (decrease)	3.6* (increase)
RASA1	BC007353	2.0 ^{a,*} (decrease)	6.5* (increase)
Uteroferrin	M30284	2.6* (decrease)	7.0* (increase)
Uteroglobulin	X59875	7.0* (decrease)	33.9 [‡] (increase)
Enigma	BC067806	2.4* (increase)	2.4* (decrease)

On each day of sampling (day 4 and day 6 post-ovulation; day 4 PO, day 6PO) mifepristone-treated animals were compared with control animals samples on the same day to derive the mean fold change in gene expression. * $P < 0.05$, [†] $P < 0.01$, [‡] $P < 0.005$, [§] $P < 0.001$ between two treatment groups on respective days based on non-parametric analogue of ANOVA followed by Mann-Whitney U test.

^aGene expression statistical system for arrays with repeated measures using mixed model method did not identify the change as significant. Table 1 shows the sample details. Gene products shown in bold were subjected to validation test using real-time RT-PCR as shown in Table 4. Twelve gene products shown in italics have been reported earlier by other groups to display differential expression in progesterone-dominated endometrium as shown in Table 6. Twenty-eight gene products showed differential expression between receptive and non-receptive endometria.

CSF1 and IL6 are known to be regulated by progesterone and possess functional linkage with the process of blastocyst implantation (Pollard *et al.* 1987, Pollard 1997, Sengupta *et al.* 2003). Leptin receptor is a single-transmembrane-domain receptor of cytokine

receptor family and related to IL6ST signal transducing component of IL6 and LIF receptors (Nakashima *et al.* 1997). This family of peptides has been shown to be involved in the process of mammalian blastocyst implantation (Gonzalez *et al.* 2000a,

Table 3 Primers and probes used in the real-time PCR validation of array data.

Candidate gene (GenBank ID)	Sequence (5' to 3')	
	Primer (as/s, T_m)	Probes (T_m)
Actin- β (X00351)	TCTCCTTAATGTCACGCACGATT (as, 59.1)	CGGCTACAGCTTACCACCACGGC (68.9)
Leptin receptor (U43168)	GACTACCTCATGAAGATCCTCACC (s, 68.9)	TGGTGAAGCCACTGCCTCCATCCA (68.0)
	CATAGCGAATCTGGAATTGAAGGT (as, 61.2)	
Uteroglobin (X59875)	ACCAACATGTGTCCTTCCTGATT (s, 61.0)	TGCCATGCTTTGAGTCCACGCCCA (68.0)
	TGCTTGTGGTTTATTGAAGAGAGC (as, 61.2)	
CSF1 (NM_000757)	CCCAACTGCTCCAGCCTCT (s, 64.5)	CTCCCTCATCAGCCCCCTCGCCC (71.0)
	GCCCTCAGTTCCTCAGAGT (as, 64.5)	
MIF (NM_002415)	AGCCATCCCTAGCAGTGACC (s, 64.5)	CAGCCAGTGCTCCAAGCCCGGT (70.1)
	ACATATTCTGGACCCACTCCTC (as, 62.8)	
CD44 (M59040)	TCATAGCTGACTACTTTGAGACGA (s, 61.2)	CAGTCACAGACCTGCCCAATGCCT (68.0)
	TGTATTCTCCTTTCTGGACATAGC (as, 61.2)	
KDR (NM_002253)	GCTCCACCTGAAGAAGATTGTAC (s, 62.8)	ACCAGTACGGCACCCTCAAAC (67.5)
	GTGAGAAATCTCTATCTCTCCTGTG (as, 59.5)	
MUC1 (NM_182741)	CAATACCAGTGCATGTGATGCG (s, 58.8)	ACCAGCAGCGTACTCTCCAGCCAC (67.4)
	GAGTGGTGGAGGAGCCTGAA (as, 59.2)	
Tastin (U04810)	CTTACTGAGAAGAATGCTGTGAGT (s, 59.2)	CGGTACGCTCTCAGAAACGCACGC (67.3)
	TGTATCTCTACCCCTAGCAAGA (as, 58.5)	
Enigma (BC067806)	GGCGTTGAATATTGAGCGGTG (s, 58.6)	CGCTCGGCAAACGCAGGGTCGA (69.2)
	GGTGACACACGGGAGTCTTG (as, 58.6)	
	CGGACAAAACGAGCACAGTG (s, 58.1)	

as, anti-sense; s, sense.

Cervero *et al.* 2004), and these peptides act via the JAK–STAT pathway (Gonzalez *et al.* 2000b). Our data demonstrate that transcripts for JAK–STAT in implantation-stage endometrium were affected by mifepristone treatment. In the present study, we have also observed a consistent involvement of KDR that is known to be operative in mediating the actions of the VEGF peptide family in mammalian endometrium (Krussel *et al.* 1999). It is however remarkable that the relative abundance of transcripts for IL6, leptin receptor, KDR, JAK, STAT were

actually up-regulated in response to mifepristone treatment, contrary to the expected changes based on earlier reports.

Earlier Catalano *et al.* (2003) observed that JAK1 was reduced at the transcript level in human luteal-phase endometrial explant cultures following mifepristone administration, and immunopositive protein levels of JAK1 were higher in stroma and luminal epithelium of luteal-phase endometrium as compared with other stages of the menstrual cycle. Also, there are reports

Table 4 Real-time qRT-PCR validation of selected candidate gene products.

Candidate	Median value (ranges)				Concordance correlation coefficient (r_c) ^a
	Day 4 after ovulation		Day 6 after ovulation		
	Control	Mifepristone	Control	Mifepristone	
KDR p^b	0.95 (0.4–1.8) <0.001	4.21 (2.1–7.7)	1.87 (0.6–2.5) <0.001	17.84 (9.1–33.8)	0.91
Tastin p^b	0.89 (0.3–1.4) <0.001	6.71 (4.6–10.4)	1.21 (0.4–3.4) <0.001	14.73 (9.6–38.4)	0.80
Leptin receptor p^b	0.64 (0.4–1.9) <0.001	11.10 (4.7–17.8)	0.71 (0.2–1.99) <0.001	14.47 (5.7–18.3)	0.79
MIF p^b	1.41 (0.4–2.6) <0.4	1.92 (0.4–4.1)	1.72 (0.5–3.2) <0.005	13.91 (9.1–24.3)	0.92
CD44 p^b	1.27 (0.3–2.3) <0.3	1.71 (0.3–3.1)	1.09 (0.2–1.8) <0.001	10.33 (5.1–15.7)	0.91
MUC1 p^b	1.32 (0.4–3.3) <0.3	0.89 (0.2–2.7)	3.67 (1.7–9.3) <0.001	0.72 (0.2–2.8)	0.79
CSF1 p^b	1.12 (0.4–3.5) <0.1	0.89 (0.2–1.9)	0.77 (0.2–1.9) <0.01	2.89 (1.2–5.1)	0.84
Uteroglobin p^b	1.11 (0.3–2.1) <0.02	0.07 (0.01–1.1)	1.11 (0.3–2.7) <0.001	19.1 (11.3–37.4)	0.91
Enigma p^b	0.77 (0.2–2.3) <0.001	4.32 (1.8–8.4)	1.97 (0.5–3.6) <0.01	0.33 (0.07–2.1)	0.81

^aThe estimated concordance correlation coefficient (r_c) between two methods for different gene products were within 95% of confidence limits (0.74–0.92). ^bFriedman test followed by Wilcoxon test for multiple comparison.

Table 5 Subjective scoring of immunopositive JAK1 and pSTAT3 in monkey endometrium on day 4 (D4) and day 6 (D6) after fertilization without (control, C) or with mifepristone, 2 mg/kg body weight, s.c. (M) application on day 2 after ovulation.

Compartment	Median score (ranges) ^a											
	JAK1 ^b						pSTAT3 ^b					
	D4		D6		D4		D6		D4		D6	
	C	M	C	M	C	M	C	M	C	M	C	M
Epithelium	3 (2–3)	2 (1–3)	3 (2–3)	2 (1–2)	2 (1–2)	2 (0–3)	2 (1–2)	2 (0–3)	NS	2 (1–2)	2 (0–3)	NS
Stroma	1 (0–1)	2 (1–3)	0 (0–1)	0 (0–1)	NS	1 (0–2)	0 (0–1)	1 (0–2)	NS	1 (0–1)	1 (0–2)	NS
Vascular	1 (0–1)	2 (1–3)	1 (0–1)	1 (0–2)	NS	3 (2–4)	2 (1–3)	3 (2–4)	<0.05	2 (1–3)	3 (2–4)	<0.05

NS, not significant.

^aBased on five-scale system for scoring: 0 (<5%), 1 (6–25%), 2 (26–50%), 3 (51–75%), and 4 (>75%). ^bFriedman test, $P<0.001$. ^cWilcoxon test for multiple comparison.

indicating that STAT3 activation in endometrium was associated with endometrial receptivity to implantation in rodents (Liu & Ogle 2002, Catalano *et al.* 2005, Nakamura *et al.* 2006). It appears therefore apparently contradictory that the relative abundance of transcripts for JAK1 and STAT3 were higher in anti-progestin (mifepristone)-treated endometrium in the present study.

In order to further examine this issue, we investigated immunohistochemically the protein products expression for JAK1 and activated STAT3 in endometrial samples collected on days 4 and 6 after ovulation with and without mifepristone treatment. We observed that the immunopositive signals for JAK1 and pSTAT3 were consistently higher in the epithelial compartment compared with the stromal and vascular compartments on days 4 and 6 after ovulation, while mifepristone treatment tended to reverse the profile. It is in fact conjectural whether the observed increasing trend of JAK1 and pSTAT3 proteins in stroma and vessels was indeed related to their higher whole tissue transcript profiles. It is indeed an open issue whether it is sufficiently logical to attempt to draw such a linear relationship between the two processes, namely transcriptomics and proteomics – each involving respective orders of synthesis, turn over and regulation (He 2006). Furthermore, we have reason to believe that input information in the form of relative abundance of transcript elements may not be tightly and digitally linked with the output level (that is protein expression) in a complex system (Segel 2001). As a reviewer has commented that the instance of a vascular gene expression of JAK1 and activated STAT3 being selectively altered in opposition to their expression behaviour in the epithelium of endometrial tissue with and without mifepristone treatment, as observed in the present study, illustrates the potential limitation of linear extrapolation of whole tissue transcriptomics. Although the proximate mechanism and function of increased expression of JAK1 and activated STAT3 in the vascular compartment following mifepristone treatment are unclear, it is likely that inflammatory changes are triggered in luteal-phase endometrium by mifepristone-induced vasocentric JAK1–STAT3 pathway (Bromberg 2001, Ni *et al.* 2003), thereby rendering endometrial hostility to blastocyst implantation. This observation correlates well with the earlier observation that early luteal-phase administration of mifepristone resulted in significant changes in mid-luteal-phase endometrial vascular physiology along with higher expression of LIF and TGF- β in vascular compartment associated with phenotypic hostility of endometrium towards blastocyst implantation (Ghosh *et al.* 1998).

Our observation that relative transcripts abundance for CD44 and MIF were higher almost to the same extent in endometrial samples only on day 6 following mifepristone application appears intriguing. However, it remains to be studied whether CD44 and MIF

Figure 2 Photomicrographs of immunohistochemical staining for JAK1 (A–F) and pSTAT3 (G–L) in endometrial tissue samples collected on day 4 after ovulation from control (A, C, D, H, K, and L) and mifepristone-treated (B, E, F, G, I, and J) monkeys. Immunostain controls (C, F, I, and L) included staining without primary antibody against JAK1 (C), pSTAT3 (I) and respective secondary antibodies (F and L). Bars = 10 μ m; gl, gland; bv, blood vessel; le, luminal epithelium.

are linked with the process of endometrial hostility or compensatory adjustment. CD44 is an integral membrane glycoprotein having two major ligands, hyaluronic acid and osteopontin. It has been suggested that 'hyaladherin' function of CD44 is linked with lymphocyte homing, cell aggregation and growth, while CD44 ligation with osteopontin mediates chemotaxis and invasion (Weber *et al.* 1996, Afify *et al.* 2006). Interestingly, CD44 expression increases in the mid-secretory human endometrium (Yaegashi *et al.* 1995, Kar *et al.* 2004). MIF is a multifunctional and widely distributed protein seemingly involved in several biological activities, especially in processes that are involved in pro-inflammatory physiological mechanism (Hagemann *et al.* 2007, Vigan *et al.* 2007). It is notable that such cellular functions that include angiogenesis, cell aggregation and migration, cell growth and pro-inflammatory changes are markedly evident in the processes related to endometrial receptivity, blastocyst implantation and placentation in the human (Bischof 1991, Ghosh & Sengupta 1998, 2004, Bischof & Campana 2000), and that the actions of some of these gene products have functional associations with steroid hormone actions in a number of systems (Dupont & Toussaint 1992, Benson *et al.* 2004).

Uteroglobin is an anti-inflammatory protein secreted by epithelial cells and its expression in the human uterine endometrium is regulated by progesterone (Becker *et al.* 1998, Beier & Beier-Hellwig 1998, Muller-Schottle *et al.* 1999). Blockade of progesterone action in endometrial cells by mifepristone led to marked up-regulation of uteroglobin gene expression in 'non-receptive' endometrium of rhesus monkeys compared with 'receptive' endometrium. Higher expression of uteroglobin and follistatin-like 1 in dysregulated non-receptive endometrium is likely to reflect the pro-inflammatory type of endometrial response that has been documented following early luteal mifepristone treatment (Ghosh *et al.* 1996, 1997, Sengupta *et al.* 2003, Miyamae *et al.* 2006). Furthermore, it would be interesting to explore whether so-called tumour suppressor-like action of uteroglobin (Zhang *et al.* 1999) has any bearing on endometrial receptivity and hostility.

In conclusion, we report here that a cohort of 28 gene products were affected in implantation-stage endometrium following early luteal-phase administration of mifepristone and those were not reported to be affected in earlier array-based studies using the model of comparative study between pre-receptive and receptive

Figure 3 Photomicrographs of immunohistochemical staining for JAK1 (A–D) and pSTAT3 (E–H) in endometrial tissue samples collected on day 6 after ovulation from control (A, C, F, and H) and mifepristone-treated (B, D, E, and G) monkeys. Bars = 10 μ m; gl, gland, bv, blood vessel.

stage endometria. Although most of the novel gene products that were observed to be affected in the present study were already known to be involved in endometrial functionality from previous studies (see Table 7 for details), we are reporting for the first time that these were differentially regulated by high-affinity antiprogesterin resulting in implantation failure in the rhesus monkey. Additionally, the observation in the present study that while a cohort of gene expression was consistent between days 4 and 6 after ovulation in a fecund cycle, a group of genes showed specific repression and derepression in the time course makes us to assume that endometrial receptivity in the rhesus monkey is associated with categorical reprogramming in endometrial transcriptomics as observed in human endometrial fibroblasts *in vitro* (Brar *et al.* 2001), human placental villous cytotrophoblasts *in vitro* (Aronow *et al.* 2001), and mouse decidual cells (Kashiwagi *et al.* 2007). We believe that elucidation of the time course pattern of endometrial receptivity associated transcriptomic

networks will be beneficial to clinical sciences in improvising strategies to assist the establishment of pregnancy. To this end, it becomes imperative to further perform large-scale transcriptomic studies using pre-receptive, receptive and non-receptive endometrial samples from fecund cycles.

Materials and Methods

General procedures and treatment groups

Healthy, mature and proven fertile male and female rhesus monkeys (*Macaca mulatta*) housed in a semi-natural condition at the Primate Research Facility of the All India Institute of Medical Sciences (New Delhi, India) were used in this study. The details of animal selection, housing, management, monitoring of cycles, mating, laparotomy, endometrial sampling, serum collection and immunoassays for serum oestradiol-17 β and progesterone have been described elsewhere (Ghosh & Sengupta 1992, Ghosh *et al.* 1993, 1996). The study design was approved by the Ethics Committee for the Use of Primates in Biomedical Research of the All India Institute of Medical Sciences.

Female monkeys showing at least two consecutive ovulatory menstrual cycles of normal length (26–32 days) were allocated to one of two groups. Animals ($n=36$) were allowed to cohabit with proven fertile males during days 8 to 16 of their menstrual cycles. Vaginal smears were checked daily for the presence of spermatozoa. Female monkeys of groups 1 and 2 were injected with either 2 ml vehicle (1:4, benzyl benzoate:olive oil, v/v; $n=18$), s.c. only (groups 1a and 2a), or a single dose of mifepristone (2 mg/kg body weight; $n=18$), s.c. in the same volume of vehicle (groups 1b and 2b) on day 2 after ovulation in mated cycles. The days of ovulation were assessed from the profiles of oestradiol-17 β and progesterone in peripheral serum samples as described earlier (Ghosh & Sengupta 1992, Ghosh *et al.* 1993, 1996). Steroid immunoassays were performed using methods described elsewhere (Ghosh & Sengupta 1992, Ghosh *et al.* 1993, 1996). Two females failed to show clear indication of ovulation and they were removed from the study. Endometrial samples were collected on days 4 (group 1) and 6 (group 2) after ovulation only from mated, ovulatory cycles that had yielded preimplantation-stage embryos (morulae and blastocysts) on uterine flushing as described elsewhere (Ghosh *et al.* 1997, 2000). The six animals who failed to yield preimplantation-stage embryo were excluded from the study. Table 1 gives the details of different treatment groups from finally selected animals ($n=28$). Recovered embryos were assessed for their developmental status under stereo-zoom microscope as described earlier (Sengupta *et al.* 1989) and were employed in different studies (Ghosh *et al.* 1997, 2000, Lalitkumar *et al.* 2005a).

Tissue collection and processing

The collection and processing of endometrial samples were performed according to the method given elsewhere (Ghosh & Sengupta 1992, Ghosh *et al.* 1993, 1996).

Table 6 Common gene products with differential expressional levels in endometrial tissue during receptivity between previous array-based studies on human and monkey models and the present study.

Reference	Subject (no. of gene targets)	Experimental design (sample size)	Common gene products as compared with present study
Kao <i>et al.</i> (2002)	Normally cycling human (~12 000)	Differential array analysis between late proliferative phase, cycle days 8–10 ($n=4$) and mid-secretory phase LH 8–10 ($n=7$)	TGF- β
Carson <i>et al.</i> (2002)	Fertile human (~12 000)	Differential array analysis between early luteal LH 2–4 ($n=3$) and mid-luteal LH 7–9 ($n=3$) phases	JunB, PGF-R, TGF- β , prolactin receptor
Borthwick <i>et al.</i> (2003)	Normally cycling human (~60 000)	Differential array analysis between proliferative phase, cycle days 9–11 ($n=5$) and secretory phase LH 6–8 ($n=5$)	α -1 anti-trypsin, JUNB, MMP3, MIF, TGF- β
Catalano <i>et al.</i> (2003)	Human endometrial explant culture (1000)	Differential array analysis between mid-secretory phase ($n=5$) endometrial explants treated with 10^{-9} M oestradiol plus 10^{-7} M medroxy-progesterone acetate and 10^{-9} M oestradiol, 10^{-7} M medroxy-progesterone acetate plus 10^{-6} MRU486	JAK1
Riesewijk <i>et al.</i> (2003)	Normally cycling human (~12 000)	Differential array analysis between paired samples ($n=5$) collected during pre-receptive LH 2 and receptive LH 7 phases	α -1 anti-trypsin, Follistatin-like 1
Ace & Okulicz (2004)	Ovariectomized hormone simulated rhesus monkey (~12 000)	Differential array analysis of normal proliferative, day 13 ($n=3$) and mid-secretory days 21 ($n=3$) and day 23 ($n=3$) endometrial samples	MMP3, TGF- β , Uteroglobin
Talbi <i>et al.</i> (2006)	Normally cycling human (~55 000)	Differential array analysis of samples collected during mid-late proliferative ($n=5$), early ($n=3$), mid ($n=8$) and late ($n=6$) secretory phases of cycle	MUC1, WIF, TGF- β , prolactin receptor
Catalano <i>et al.</i> (2007)	Normally cycling human (custom made; 16 000)	Differential array analysis of LH+8 endometrial samples with no treatment (control; $n=15$) and with mifepristone (200 mg) treatment at either 6 h ($n=5$) or 24 h ($n=4$)	Follistatin-like, MMP3, MMP10
Sherwin <i>et al.</i> (2007)	Adult female baboons (custom made; 8000)	Differential array analysis of LH+10 samples with either no treatment (control; $n=2$) or rhCG (1.25 IU/h) treatment ($n=2$) during days 5–10 after ovulation	IL6

Briefly, maximally retrievable endometrial samples were collected on days 4 and 6 after ovulation from animals of different treatment groups (Table 1) following laparotomy and fundal hysterotomy under ketamine (12 mg/kg body weight; Vetlar, Parke-Davis, Mumbai, India) anaesthesia. Tissue samples were quickly washed in ice-cold PBS to get rid of blood and debris and promptly employed for RNA extraction. An arbitrarily selected small piece from each sample was processed for chemical fixation and preparation of paraffin blocks that were used for immunohistochemistry as described earlier (Ghosh *et al.* 1998).

RNA extraction

Total RNA was extracted using Trizol (Agilent Technologies Singapore Pvt. Ltd, Shung Avenue, Singapore) and cleaned up with DNase I (Sigma Chemical Co.) and subjected to re-extraction when it was necessary. The yield and purity of the extracted RNA was checked using standard protocols of absorbance ratio between 260 and 280 nm and 1% agarose gel electrophoresis (Farrell 1998). Furthermore, RIN score of individual sample was determined using Agilent 2100 Bioanalyzer, RNA 6000 Nano LabChip kit and Agilent 2100 Expert Software (Agilent Technologies, Inc., Santa Clara, CA, USA) according to a methodology described by Schroeder *et al.* (2006). Three samples that could not yield either sufficient

purity or adequate amount or RIN score above 7 were discarded (Table 1). All accessory chemicals were purchased from Sigma Chemical Co. and Invitrogen (Agilent Technologies Singapore Pvt. Ltd).

Nylon array

cDNA arrays were produced containing custom-tailored 409 sequence verified clones spotted in duplicate (15 ng each in 200 nm) in 576 spots and u.v. cross-linked on a 6×6 cm nylon membrane along with the quality assurance by the Clontech (Takara Bio Inc., Shiga, Japan). Details of the gene products examined in the present study are given in Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental/.

Complex probe synthesis and array hybridization

The complex probe synthesis, hybridization and post-hybridization stringency wash were performed according to Catalano *et al.* (2003). Briefly, radiolabelled cDNA probes were synthesized by labelling 5 μ g total RNA with [α - 32 P]dATP (Bhava Atomic Research Centre, Hyderabad, India) followed by the removal of unincorporated radioactivity on columns using a kit obtained from Clontech. cDNA probes were denatured and subjected to hybridization at 65 °C for 16 h in

Table 7 Genes showing differential display in the present monkey study and reported to be functional in endometrium in previous reports.

Functional classification, Gene name	Reference
Homeobox Distal less homeobox 4 Enigma	Quinn <i>et al.</i> (1998a, 1998b)
Transcription/translation associated/housekeeping factors <i>JUN</i> Elongation factor 1- α <i>JUNB</i> Ribosomal protein S7 Ribosomal protein S9 <i>Wif</i>	
Cytokines Colony stimulating factor 1 <i>EGFR</i> Follistatin-like 1 Interleukin 6 <i>TGFB1</i> Tumour necrosis factor α	Pollard (1997) Harris <i>et al.</i> (2005) Sengupta <i>et al.</i> (2003) Ghosh <i>et al.</i> (1998) Lalitkumar <i>et al.</i> (2005a, 2005b)
Receptors Kinase domain receptor Leptin receptor Prostaglandin F receptor Prolactin receptor	Krussel <i>et al.</i> (1999) and Critchley <i>et al.</i> (2006) Cervero <i>et al.</i> (2004) Milne & Jabbour (2003) Tseng & Mazella (1999)
Signalling factors <i>JAK1</i> <i>STAT3</i> <i>RASA1</i>	Ghosh <i>et al.</i> (2006) Catalano <i>et al.</i> (2005) and Ghosh <i>et al.</i> (2006) Scambia <i>et al.</i> (1993)
Enzymes MMP3 MMP9 MMP10 3- β -hydroxysteroid dehydrogenase Cyclooxygenase 1 17, 20 desmolase	Salamonsen & Woolley (1996) and Slayden & Brenner (2006) Rawdanowicz <i>et al.</i> (1994) Rawdanowicz <i>et al.</i> (1994) Seki <i>et al.</i> (1987) and Rhee <i>et al.</i> (2003) Kim <i>et al.</i> (1999) and Marions & Gemzell-Danielsson (1999) Seki <i>et al.</i> (1987) and Matsuzaki <i>et al.</i> (2000)
Keratan sulphotransferase	
Adhesion molecules Bystin CD44 Integrin α -6 Mucin 1 Tastin	Aoki & Fukuda (2000) Yaegashi <i>et al.</i> (1995) Rai <i>et al.</i> (1996) Aplin <i>et al.</i> (1998) Suzuki <i>et al.</i> (1999) and Aoki & Fukuda (2000)
Mediator factors α -anti-trypsin MIF Tissue factor, thromboplastin Thioredoxin Uteroferrin Uteroglobin	Marshall & Braye (1987) Vigan <i>et al.</i> (2007) Schatz <i>et al.</i> (2000) and Kato <i>et al.</i> (2005) Maruyama <i>et al.</i> (1997) Gonzalez <i>et al.</i> (1994, 1995) Muller-Schottle <i>et al.</i> (1999)

hybridization buffer at 1×10^6 c.p.m./ml with the array membrane treated previously with pre-hybridization buffer. The details of hybridization buffer are given elsewhere (Mooney *et al.* 2000). The hybridized membranes were subjected to step-wise stringency wash in SSC buffer and

exposed to low-energy storage phosphor screens (Bio-Rad Laboratories) for 48 h and scanned at 50 μ m resolution in a Bio-Rad Molecular Image Fx Scanner (Bio-Rad Laboratories).

Array analysis

Hybridization signals were quantified using ProArray Analysis software (Bio-Rad Laboratories). Analysis of data retrieved from separate membranes with the same RNA samples yielded QC statistics highly concordant with that of the manufacturer, and it revealed more than 95% confidence level. In order to screen out biological variation from technical variability, integrated signal analysis for a given membrane was done and signal spots that were low after averaging as compared with background values were removed. Data normalization, averaging, calculation of relative abundance of transcripts, ratio analysis and fold changes were performed on log-transformed data using Arrayassist (Stratagene, North Torey, La Jolla, CA, USA) and GeneSpring (Agilent Technologies). Statistical analysis of data showing more than twofold changes were performed using non-parametric analogue of ANOVA followed by Mann-Whitney *U* test to assess the statistical significance of differential expression between groups at $P < 0.05$ (Claverie 1999). As suggested in a recent review (Horgan 2007), we have also employed an additional, independent and suitable statistical analysis method with multiple testing correction (mixed model method) having type I error rate set at < 0.01 (Pan 2002) using Gene Expression Statistical System (GESS, Kaysville, UT, USA) version 2006 for significance analysis of microarrays (<http://www-stat.stanford.edu/~tibs/SAM>) followed by filtering for more than twofold difference and it was employed to test the power of the results obtained from conventional non-parametric statistical analysis as mentioned above.

Quantitative real-time RT-PCR

The relative expression of ten selected genes inclusive of actin- β as the endogenous control in endometrial samples collected from control and mifepristone treated animals were performed and compared using real-time RT-PCR technology on a Bio-Rad platform (iCycler iQTM Real time PCR detection system, Bio-Rad). β -Actin was selected as an endogenous control based on its observed expressional consistency in arrays on data analysis. Primers and labelled probes were designed on Beacon Designer software (Labware Scientific Inc., Milipitas, CA, USA) and obtained from Qiagen. Details of primers and probes for target genes are given in Table 3. An optimized kit (Quantitect multiplex PCR kit, Qiagen) was used according to the protocol given by the manufacturer. Briefly, cDNA was synthesized with Superscript II from same total RNA (5 μ g) samples that were used for the array experiments and levels of select transcripts were estimated by TaqMan analysis for each cDNA sample. Cycle threshold (C_t) values were obtained and ΔC_t values between experimental C_t and normalization C_t were determined and relative expression ratios between groups were determined using a method described by Pfaffl *et al.* (2002). Statistical analyses of Q-RT-PCR data from all groups were done using

Friedman test followed by multiple comparison test based on rank sums Wilcoxon test (Hollander & Wolfe 1999) using NCSS software version 2007 (Kaysville, UT, USA).

Lin's concordance correlation coefficients (r_c) between values of transcript abundance obtained from arrays and quantitative RT-PCR for nine selected genes were estimated (Lin 1989) to assess the reproducibility assurance of signal estimation from arrays.

Immunohistochemistry

Endometrial samples were collected from mated rhesus monkeys yielding preimplantation stage embryos on days 4 and 6 after ovulation following s.c. administration of vehicle (control) or mifepristone (2 mg/kg body weight) as described above. Immunohistochemical staining for detection of JAK1 and activated STAT3 were performed using specific antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) according to the methods provided by the manufacturer. Briefly, deparaffinized and hydrated tissue sections were subjected to microwave heating in 0.1 M sodium citrate buffer (pH 6.0) for retrieval of antigens. The endogenous peroxidase activity was quenched and non-specific binding was blocked with non-immune sera as described earlier (Ghosh *et al.* 1998, Sengupta *et al.* 2003). Visualization was achieved using Vectastain ABC Peroxidase Elite Kit (Vector Laboratories, Burlingame, CA, USA) and freshly prepared 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Company) as substrate. All immunostaining procedures were performed in a single run along with controls (antibody omission, replacement and neutralization). Immunohistochemically stained sections were subjectively scored for different compartments using a Leica microscope and a standardized five-scale system: 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), 4 (>75%) as described elsewhere (Sengupta *et al.* 2003). Statistical analyses of the scores were done using Friedman test followed by multiple comparison test based on rank sums Wilcoxon test (Hollander & Wolfe 1999) using NCSS software version 2007.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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