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REVIEW

Large-scale gene expression studies of the endometrium: what have we learnt?

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Abstract

The endometrium is a dynamic tissue that undergoes coordinated changes under the influence of steroid hormones. This results in proliferation and differentiation culminating in a receptive state, followed by menstruation and endometrial repair. These functions involve complex interactions between the epithelium, stroma and leucocytes in the endometrium. Understanding the underlying causes of endometrial disorders, such as infertility, endometriosis and heavy menstrual bleeding, therefore represents a considerable challenge. Recently developed techniques, such as differential display and DNA microarrays permit the abundance of thousands of mRNA transcripts within cells or tissues to be measured simultaneously. This provides a new approach to understanding the complex interactions that underlie both healthy and disease states. Responses of the endometrium to hormones or drugs can be studied and the response of the system as an integrated whole can be assessed. Comparisons of endometrium from healthy women and those with endometrial dysfunction have advanced our understanding of key areas of endometrial physiology, including infertility, receptivity, endometriosis and cancer. Using this approach, novel genes controlling specific endometrial functions like receptivity have been identified for functional testing. This paper will review the impact of these techniques for transcript profiling on our understanding of selected areas of endometrial biology and discuss the potential applications in future.

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Introduction

In preparation for embryo implantation, endometrium responds to ovarian sex steroids by undergoing a dynamic series of morphological, biochemical and molecular changes. The endometrium is a complex tissue with distinct cellular compartments. Luminal epithelium provides a defensive barrier and is also the site of embryo attachment. The glandular epithelium secretes paracrine and autocrine factors and the stroma undergoes decidualisation to support and regulate trophoblast invasion. The many cell types within these compartments (epithelial, stromal and endothelial cells as well as leukocytes) are influenced not only by the hormonal milieu, but also by paracrine messengers secreted by the neighbouring cells. Genomic techniques have provided a powerful set of tools for understanding the transcriptional changes that occur in this complex tissue. They have permitted the investigation of both normal cyclical changes as well as the study of disease states, such as menorrhagia, endometriosis and endometrial cancer. This paper will review how these techniques have been used so far to study endometrial biology and what they have revealed, with particular emphasis on the studies of endometrial receptivity.

Several genomic techniques have been developed to identify differential mRNAs, expressed at different levels between the two RNA samples. Estimates vary as to how many of the projected 30 000 human genes are expressed in any one cell type, but in most tissues, 50% of mRNA by mass is comprised of only 300–500 of the most abundant genes (Bishop *et al.* 1974, Axel *et al.* 1976). Therefore, when choosing a genomic technique to interrogate endometrial gene expression, the sensitivity of the technique (ability to differentiate between two transcriptomes that vary only in low-abundance transcripts) as well as the reproducibility, cost and ease of use, must be considered. Examples of the most commonly used genomic techniques are given in Table 1. Of these possible approaches, differential display (DD)-based methods and more recently, DNA microarrays have been most widely used in the study of endometrial biology.

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Table 1 Classification of genomic techniques.

Classification	Examples
Hybridisation techniques	
Differential colony hybridisation	Colony filters
Nylon arrays	PCR product macro/ microarrays
Glass arrays	Microarrays, oligonucleotide arrays
Subtractive techniques	
Subtractive hybridisation	Suppression subtractive hybridisation
Representational difference analysis	
Enzymatic degrading subtraction	
Gel based techniques	
RNA arbitrarily primed (RAP-PCR)	
Differential display (DD)	Profiling, indexing, gene expression fingerprinting
Sequencing based techniques	
Expression sequence tags	
Serial analysis of gene expression (SAGE)	

DD-based techniques

The general principle of DD methods is to amplify partial cDNA sequences from a subset of mRNAs, by RT and PCR (Liang & Pardee 1992). cDNA synthesis is performed using a set of oligo-dT nucleotides that have at their 3′ end, two other bases (e.g. ttttttttttca). These oligonucleotides anchor the polyadenylate tail of a subset of 1/12th of all the mRNAs. PCR amplification of this subset is performed using short primers (6–7 nucleotides) of arbitrary sequence, to bind upstream of the poly-A tail. This results in radiolabelled-cDNA fragments of varying lengths that can then be resolved by gel electrophoresis and visualised by autoradiography. Side-by-side comparison of mRNA species from two or more related samples allows the identification of cDNAs that are up- or downregulated. Variations of the original DD technique have been developed, with the aim of reducing the number of false-positive candidates. One of these technical variations is indexing, which involves the synthesis of short cDNA termination products by PCR amplification, using a set of oligonucleotide primers, in multiple reactions (see Figs 1 and 2). This produces a subset of 10–60 bands of amplified cDNAs (the profile or index), allowing a comparison between two mRNA populations. The false-positive rate using this technique is said to be greatly reduced compared to the conventional DD (Mahadeva *et al.* 1998).

A strength of DD is that it allows novel transcripts to be identified, but it suffers from a high rate of false positives (Liang & Pardee 1995, Sompayac *et al.* 1995), as well as a bias towards high copy number mRNAs (Bertioli *et al.* 1995). This may make DD an inappropriate choice of technique in systems, where only a few mRNA species are expected to differ (Sompayac *et al.* 1995). However, a number of endometrial biologists have successfully used this technique (Table 2). Okulicz identified several progesterone-regulated genes whose expression in primate endometrium is limited to the window of implantation (Okulicz *et al.* 2003). This experiment highlights the strength of DD in that half of the genes identified were novel expression sequence tags. Several new groups of

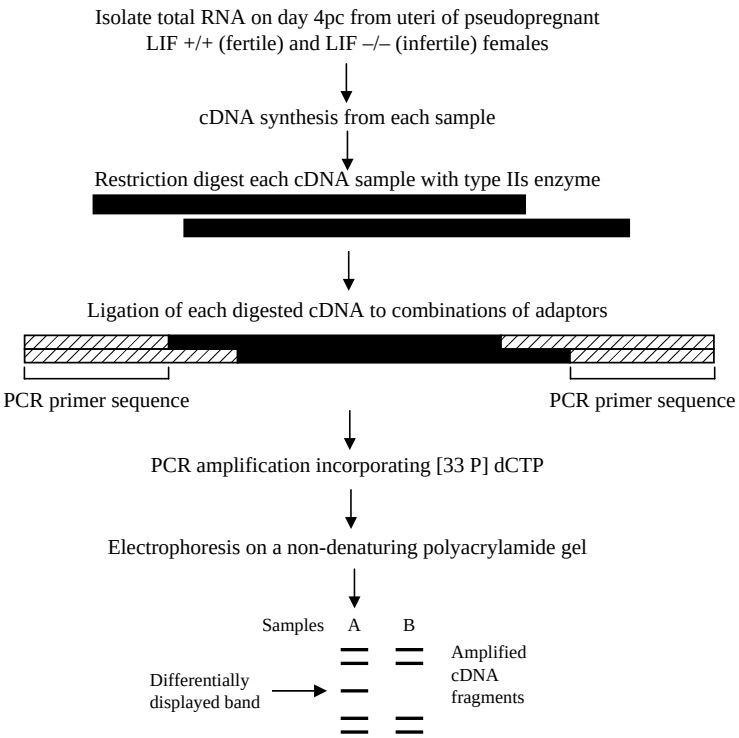

Figure 1 Schematic representation of the steps involved in a cDNA indexing study to compare RNA samples from two different tissues.

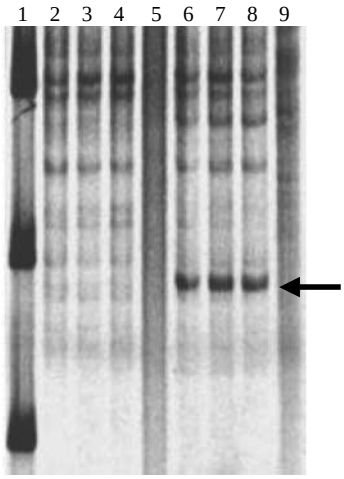

Figure 2 cDNA indexing comparison of the uterine transcriptome, at 96 h of pseudopregnancy, between a leukaemia inhibitory factor (LIF) wild-type (LIF +/+) animal and a LIF null mutant (LIF −/−) animal. Aliquots of double-stranded cDNA, digested with FOK1, a type-II restriction enzyme and ligated to a mixture of 32 adaptors, was amplified by PCR, in which ^{33}P was incorporated. The amplified products were electrophoresed and transferred on to a filter paper. The dried gels were exposed to phosphorimager screens and then X-ray film. Three aliquots of each adaptor ligated cDNA sample were amplified and electrophoresed. A single aliquot of minus-RT 'cDNA' (cDNA 'synthesised' without reverse transcriptase) was amplified to act as a control for genomic contamination of each cDNA sample. Results from amplification with a single primer pair are shown. A clear, differentially expressed band was identified which is upregulated in LIF−/− uterine cDNA.

genes were identified and shown to be regulated by progesterone in endometrium. Similarly, Nie and colleagues. in a series of experiments using DD (Nie *et al.* 2000*a*,*b*,*c*), used it to compare the endometrial gene expression on day 4 of pregnancy, at implantation and intersite locations in the mouse. This led to the identification of the calbindin family of proteins, as being specifically upregulated at the site of embryo attachment. In subsequent experiments, they showed that functional blockade of the calbindins, CaBP-d9k and CaBP-d28k, successfully prevents implantation (Nie *et al.* 2000*c*, Luu *et al.* 2004). These regulators of calcium homeostasis clearly play an important role in the process of implantation. Other genes identified in this series of mouse experiments included proprotein convertase-6 (PC6), an endoprotease that cleaves inactive precursors into active molecules (Nie *et al.* 2003). PC6 was shown to be expressed in the stroma beneath the implantation site in the mouse and when expression was inhibited by anti-sense morpholinos, complete functional blockade of the implantation process was achieved. These examples show that genes with important functions in endometrial biology can be identified using this technique. However, DD is slow and labour intensive and does generate a high number of false-positive candidates. More recently, DD has been superseded by other techniques, such as suppression subtractive hybridisation and microarray-based investigations. Selected examples of these techniques and their use in endometrium are given in Table 2.

Array-based studies

Array-based expression studies followed from the work of Southern and others (Gillespie & Spiegelman 1965, Southern 1974) requires the hybridisation of complementary strands of DNA or RNA onto an ordered grid of DNA, which is arrayed on a glass or nylon support. The arrayed DNA sequences are usually mechanically spotted and are either oligonucleotides between 20 and 80 bp or cDNAs of several hundred base pairs. In some cases, the DNA arrays are manufactured by synthesising oligonucleotide spots *in situ* onto the glass surface of the array. Fluorescent or radiolabelled complex probes are prepared from mRNA, isolated from cells or tissues of interest. After hybridisation of these complex probes to the array, the signal generated by the hybridisation of each specific cDNA species, to its complementary array sequence, can be measured with a confocal laser scanner. cDNAs from two sources can also be compared by labelling complex probes from the two samples with different colour fluorophores (such as Cy3-green and Cy5-red). These probes can then be hybridised simultaneously to the same microarray and the signal from each hybridised fluoroprobe measured. One of the major hurdles in the use of microarrays is the analysis of the raw hybridisation data (Gaasterland & Bekiranov 2000). Appropriate mathematical transformation to normalise the data is essential to account for experimental and biological variation. This is one of the major causes of error and difference between laboratories, since the statistical approaches used can greatly affect the outcome. If the same raw data from an array experiment is analysed, using different statistical methods, there can be major differences in the number of genes identified as altered.

The results of microarray experiments are most frequently reported as a fold difference; for example, a gene transcript may be reported as increased threefold in expression level between secretory and proliferative endometrium. Such an approach can be highly misleading as hybridisation signals obtained for genes expressed at low levels show greater variation than genes expressed at high levels due to experimental error and background noise. Therefore, a tenfold change in expression of a gene expressed at low levels may be less easily validated in subsequent experiments than a gene that shows a twofold change,

Table 2 Examples of large scale endometrial gene expression studies.

Biology studied	Technique used	No. of genes studied	Biological material used	Reference
Endometriosis	Atlas Nylon array	597	Primary stromal cells treated with IL-1β	(Lebovic *et al.* 2002)
	Affymetrix array	12 686	Eutopic endometrial biopsies from women with and without endometriosis	(Kao *et al.* 2003)
	Nylon array	4133	Eutopic vs ectopic biopsies	(Eyster *et al.* 2002)
	Research genetics cDNA array	9600	Eutopic vs ectopic biopsies	(Wu *et al.* 2006)
	Glass slide cDNA array	23 000	Eutopic vs ectopic biopsies	(Arimoto *et al.* 2003)
Endometrial receptivity	DD		Mouse implantation and intersite	(Nie *et al.* 2000*b*)
	DD		Receptive vs non-receptive primate endometrium	(Okulicz *et al.* 2003)
	Subtractive hybridisation		Rat implantation and intersite	(Simmons & Kennedy 2002)
	Affymetrix array	10 000	Implantation and intersite from day 4 mice Delayed implantation with or without P_4	(Reese *et al.* 2001)
	Affymetrix array	6500	Uteri from day 3.5 and day 5 mice	(Yoshioka *et al.* 2000)
	Affymetrix array	6000	Uteri from mice treated on day 3 with RU486 or control	(Cheon *et al.* 2002)
	Custom array	5000	Ovariectomised cynomologus monkeys, treated with oestrogen and RU486	(Tynan *et al.* 2005)
	Custom nylon array	1000	Endometrial explants treated with RU 486	(Catalano *et al.* 2003)
	Affymetrix array	60 000	Pooled endometrial biopsies	(Borthwick *et al.* 2003)
	Affymetrix array	12 000	Five paired endometrial biopsies at LH +2 and LH+7 from the same women	(Riesewijk *et al.* 2003)
	Atlas Nylon array	1176	Endometrial cell lines (RL-95-2 and HEC-1-A) and endometrial biopsies LH+2 vs LH+7	(Martin *et al.* 2002)
	Atlas Nylon array	375		(Dominguez *et al.* 2003)
	Affymetrix array	12 000	Three early (LH+2-4) and three mid secretory (LH+7-9) endometrial biopsies	(Carson *et al.* 2002)
	Affymetrix array	12 686	Endometrial biopsies (10 late proliferative phase and 18 from LH+8 to LH+10)	(Kao *et al.* 2002)
	Affymetrix array	12 000	Endometrial biopsies from day 13 and days 21–23 in Rhesus monkeys	(Ace & Okulicz 2004)
Endometrial and trophoblast interactions	DD		Stromal cells cultured with BeWo cell line	(Cowan *et al.* 1999)
Decidualisation	Atlas Nylon array	588	Primary culture of stromal cells treated with progesterone and cAMP	(Popovici *et al.* 2000)
	Affymetrix array	12 686	Primary culture of stromal cells treated with cAMP	(Tierney *et al.* 2003)
	Atlas array	1176	Primary culture of stromal cells	(Okada *et al.* 2000)
	Atlas array	1000	Primary culture of stromal cells treated with progesterone	(Okada *et al.* 2003)
	Custom microarray	9600	Decidua and first trimester trophoblast	(Chen *et al.* 2002)
	Incyte GEM-V array	6918	Primary culure of stromal cells	(Brar *et al.* 2001)
Endometrial cancer	Atlas Nylon array	588	Ishikawa cell lines transfected with PRA and PRB, treated with progestins	(Smid-Koopman *et al.* 2003)
	NCI microarray	9984	Endometrial cancer biopsies	(Risinger *et al.* 2003)
	Atlas array	588	Endometrial cancer vs normal endometrium	(Meng *et al.* 2001)
	Microarray	4096	Endometrial cancer vs normal endometrium	(Zhou *et al.* 2003)
	Atlas Nylon array x2	1176 & 588	Hec50 cell line transfected with PRA and PRB, treated with progesterone	(Dai *et al.* 2002)
	Atlas Nylon array	1176	Normal endometrial epithelial cells treated with oestrogen and norethisterone	(Oehler *et al.* 2002)
	Custom array	4009	Endometrial cancer cell lines (n=14), transfected with PTEN tumour suppressor	(Matsushima-Nishiu *et al.* 2001)
	Affymetrix array	6000	Endometrial cancer vs normal endometrium	(Mutter *et al.* 2001)

but is highly expressed. Secondly, statistical significance and biological significance are not clearly related. A gene which changes tenfold in expression levels is not necessarily of more biological significance than the one which changes twofold. Interpretation of microarray data is therefore not straightforward.

The use of arrays to identify genes involved in endometrial receptivity

Successful implantation requires the simultaneous development of both, a competent blastocyst and a receptive endometrium, under the influence of ovarian

steroids. However, the molecular mechanisms by which endometrium becomes receptive remain unclear. Two approaches have been used to identify genes regulated by progesterone in the endometrium. The first has been to compare proliferative and secretory phase endometrium, to identify global gene expression changes between the oestrogen- and progesterone-dominated phases of the cycle. Kao *et al.* (2002), Borthwick *et al.* (2003) compared gene expression in late proliferative and mid-secretory endometrium. The latter study pooled RNA from five samples at each time point, whereas Kao hybridised samples individually. Both these studies used Affymetrix oligonucleotide arrays and so the results can be compared directly. Borthwick showed that 90 transcripts were significantly upregulated and 59 downregulated, between the pooled, proliferative and secretory phase samples. Using different analysis criteria, Kao and colleagues reported 116 upregulated genes and 224 downregulated (Kao *et al.* 2002). Approximately 30% of the genes were common to both studies. Differences between the lists are likely to be due to the very different methods of analysing the data generated by the same microarray platform, as well as the use of pooled vs separate samples.

A second approach has been used in two studies, where the effect on transcript expression of RU486, a progesterone receptor (PR) antagonist, has been assessed. This drug binds to the PR and blocks the action of progesterone. Cheon *et al.* (2002) compared uterine RNA isolated on day 4 of pregnancy from mice treated with RU486 and uterine RNA from normal animals. Using Affymetrix microarrays, 78 known genes were identified as downregulated more than twofold and 70 were upregulated following the RU486 treatment. Independent verification by northern blot of ten of these candidate genes, confirmed their upregulation by progesterone. Only 4 of the 70 genes upregulated by progesterone were previously known to be hormonally regulated. Secondly, some genes that are known to be on the array and progesterone regulated in murine endometrium, such as Hoxa10 and calcitonin, were not detected. The failure to detect these genes probably reflects an expression level below the detection threshold of the array.

In women, a single dose of RU486 (mifepristone) in the secretory phase of the menstrual cycle, rapidly renders the endometrium unreceptive (Danielsson *et al.* 2003). We have recently used microarrays to examine changes in endometrial gene expression following RU486 administration during the implantation window (Catalano *et al.* 2003). Pipelle biopsies taken from the endometrium at 6 or 24 h after RU486 administration on LH+8 were analysed. At 6 h after RU486, 6 genes were found to be significantly upregulated and 90 were downregulated compared to controls (Sharkey *et al.* 2005). Although RU486 can affect glucocorticoid and androgen receptor function, the majority of these genes are likely to be directly progesterone regulated. This clearly shows how microarrays can be used *in vivo*, to study drug action on the endometrium.

A major challenge when faced with these long lists of genes, regulated by progesterone is to identify which of the genes perform an essential role in implantation and which do not. Cheon *et al.* (2002) demonstrated the value of their microarray analysis by showing that leukocyte 12/15 lipoxygenase (Alox15) is essential for implantation. Alox15 was found to be upregulated by progesterone using microarrays. It acts as a lipid-metabolising enzyme that generates hydroxy-eicosatetraenoic acid (HETE), a signalling molecule, which controls cell differentiation. Cheon *et al.* (2002) showed that Alox15 is expressed in the luminal epithelium at the time of implantation. Administration of an inhibitor (AA-861) to block Alox15 activity in the uterus reduced implantation by 80% (Li *et al.* 2004). Administration of rosaglitazone, an agonist for the proliferation-activated receptor-γ (PPARγ), restored implantation in mice pretreated with AA-861. This suggests that metabolites generated by Alox15, like 12-HETE, function as activating ligands of PPARγ and thus PPARγ is a downstream target of metabolites generated by Alox15 (Fig. 3). This array-based study also identified other progesterone-regulated genes, whose activity in endometrium had previously been unsuspected. Glutathione peroxidase-3 and manganese superoxide dismutase protect against cellular damage by reactive oxygen species. Along with a number of metallothionine proteins, these molecules may protect the preimplantation embryo against damage by oxygen radicals and heavy metal toxicity.

Many studies have also been performed comparing prereceptive and receptive murine or human endometrium, with the aim of identifying a 'molecular signature' characteristic of a receptive endometrium. The murine studies have used several different paradigms to identify receptivity genes. These include comparisons of: implantation vs intersite tissue (whole uterus (Nie *et al.* 2000*b*) or luminal epithelium only (Yoon *et al.* 2004)), prereceptive vs receptive uterus (Yoshioka *et al.* 2000) and delayed vs nidatory oestrogen-treated uteri (Reese *et al.* 2001). The ability of microarrays to analyse thousands of mRNAs simultaneously provides a powerful new approach to the identification of markers of receptivity. In women, under the influence of progesterone, endometrium becomes receptive from about day LH+5 to LH+10. Morphological changes (Noyes *et al.* 1950) and more recently, molecular markers (Ponnampalam *et al.* 2004) have been identified that are expressed at different stages of the luteal phase. These provide a novel means to assess whether normal endometrial development is occurring.

Several studies have sought to identify new markers of the receptive state using array-based technology. Endometrium from patients with endometriosis or

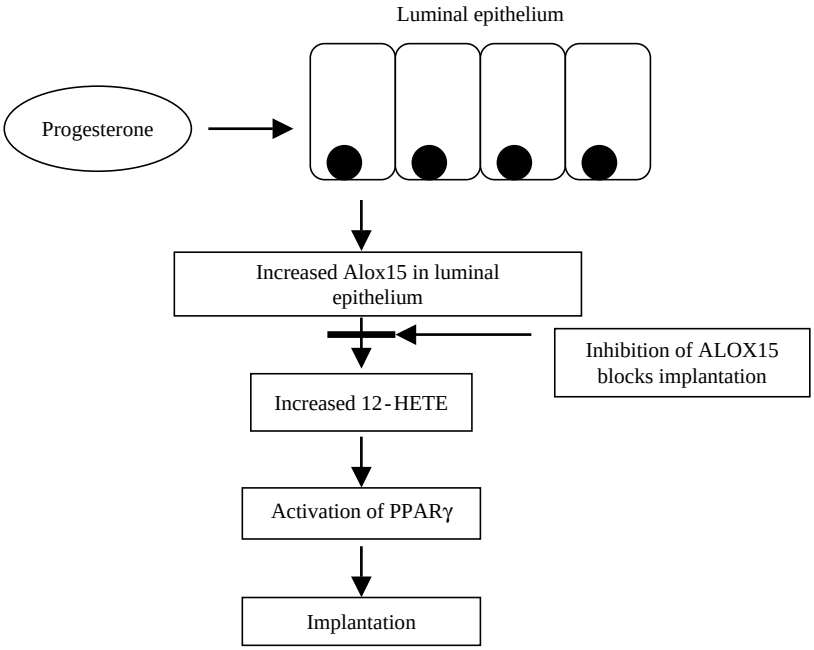

Figure 3 Using microarrays, the enzyme ALOX15 has been identified as essential for implantation in murine endometrium. ALOX15 is upregulated in the luminal epithelium by progesterone. This results in increased synthesis of 12-HETE, which in turn activates PPARγ. Inhibition of ALOX15 activity results in implantation failure.

unexplained infertility, when compared to endometrium from women with a past history of fertility, shows a number of changes in the transcriptome, which may be of functional significance (Giudice *et al.* 2002). However, there is substantial overlap in expression levels of these markers between subfertile patients and normal controls and so clinically, the predictive value of any one of these molecular markers is low (Damario *et al.* 2001). Two studies have compared gene expression in women with a past history of fertility on days LH+2 (prereceptive) and LH+7 (receptive). Carson *et al.* (2002) compared three biopsies at each time point, taken from different women, whereas Riesewijk *et al.* (2003) compared paired biopsies taken from the same women ($n=5$) at two time points. The first study identified 370 transcripts as upregulated and 323 downregulated by at least twofold, whereas Riesewijk identified 153 genes as upregulated and 58 as down-regulated by an average of threefold or more. More significantly, many new genes were identified to show regulation, a large number of which had not been previously shown to even be expressed in endometrium. For example, members of the Wnt family of signalling molecules and related inhibitors were identified for the first time in endometrium and may play a role in epithelial/stromal interactions during the receptive phase (Tulac *et al.* 2003). Indeed in mice, uterine Wnt signalling has recently been shown to be essential for implantation (Mohamed *et al.* 2005).

Although there is a substantial overlap in the gene lists generated from the array experiments examining secretory phase endometrium, they are not identical. There are a number of explanations for this and it is important to appreciate the limitations of array-based interrogation. First, in designing the study, it is necessary to select well-defined populations for comparison (in this case LH+2 and LH+7). Secondly, samples may be pooled at each time point prior to hybridisation (Borthwick *et al.* 2003) or hybridised separately (Kao *et al.* 2002). Pooling the samples reduces the cost of the experiment and gives a biological average, but reduces the power of the post-array statistical analysis and does not allow exclusion of 'unusual or untypical' samples that may reflect outliers. Subtle differences in the analysis criteria, like the choice of data normalisation methods or gene selection criteria may also account for large differences in the gene lists generated, e.g. average change of threefold (Carson *et al.* 2002) or twofold (Riesewijk *et al.* 2003). Finally, these studies involved very small patient groups ($n=3$ or 5). With such small groups, the biological variation between patients due to genotypic and environmental differences may substantially influence the final gene lists. In genetically identical inbred mice, housed under identical environmental conditions, microarray studies of kidney transcript abundance showed that 3.3% of RNA transcripts exhibited altered expression over and above what could be explained by technical variation between arrays (Pritchard *et al.* 2001). In a microarray experiment, which examined 12 000 genes, this amounts to a substantial number of false positives that may have little biological relevance. Thus, the need for rigorous and robust experimental design and careful consideration of the data analysis is essential to reduce false positives (Stafford & Liu 2003). Validation of candidate gene expression changes using northern blotting or quantitative PCR is essential. The correlation of transcript abundance change with changes in the corresponding protein, followed by functional testing of the

biological effect of that protein, allows the biological significance of the microarray changes to be confirmed.

The use of microarrays in the study of endometriosis

Endometriosis is characterised by the presence of endometrial tissue at ectopic sites, typically within the pelvis and the ovary. It is associated with pelvic pain and subfertility. A recent microarray study of gene expression in luteinising hormone (LH)-timed (LH+8 to 10) biopsies of eutopic endometrium from normal fertile women ($n=7$) and women with endometriosis ($n=8$), identified 91 upregulated and 115 downregulated genes (Kao *et al.* 2003). Functional classification of these revealed genes involved in cell adhesion, apoptosis, signal transduction and also some secreted proteins. The majority had not previously been shown to be dysregulated in endometriosis and may provide new insights into the subfertility in women with endometriotic disease that does not involve the fallopian tube or ovarian function. One such candidate is S100E, a member the calcium-binding family of proteins, whose expression is reduced in eutopic endometrium of women with endometriosis. Mice carrying a null mutation of a closely related protein, S100A8, are infertile due to an implantation failure (Passey *et al.* 1999). Downregulation of the calcium-binding protein, calbindin d9K, using antisense oligonucleotides also prevents implantation (Luu *et al.* 2004). Taken together, these results imply a critical role for calcium homeostasis in normal implantation. There is now a wealth of data studying both individual genes and using microarray methods that show differences in gene expression between normal and eutopic endometrium from women with endometriosis (reviewed in Wu *et al.* 2006). These authors performed microarray analysis of eutopic and ectopic endometrium from the same patient using samples obtained by laser capture microdissection (LCM). They identified over 100 genes with known functions that differed between the two. An unresolved question is whether the differences in eutopic endometrium are caused by the presence of ectopic endometrium in the peritoneum or whether they pre-date the establishment of endometriosis. The gene, CYR61, is one of those which show increased expression in eutopic endometrium in women with endometriosis, compared with normal women (Absenger *et al.* 2004). Using the baboon model in which endometriosis can be induced in the peritoneum, Gashaw *et al.* (2006) showed that expression of CYR61 is induced in eutopic endometrium of animals with induced endometriosis, within a month of inoculation of menstrual tissue in the menstrual cavity. This provides evidence for a feedback mechanism from the resulting lesions that can induce changes in the gene-expression patterns in the eutopic endometrium. An outstanding question, therefore, is which of the alterations seen in eutopic endometrium of women with endometriosis is induced by the presence of peritoneal lesions and which pre-date the development of endometriosis and may hence contribute to the pathogenesis of the disease.

Expression profile analyses of endometrial cancer

One of the most valuable potential uses of microarrays is their use in the classification of endometrial cancers (Mutter *et al.* 2001, Risinger *et al.* 2003). These studies have shown that microarrays provide a powerful approach for identifying therapeutic targets. They have also generated a list of candidate biomarker genes that provide discrimination between normal and malignant tissues, and different tumour types. Endometrial cancers are characterised by mutations of PTEN, KRAS2 and CTNNB1 (type I) or TP53 and Her-2/neu (type II), although as 50% of endometrial cancers lack these mutations, it is likely that there are unrecognised pathways that are mutagenic. Holland *et al.* (2004) used microarrays to identify peroxisome proliferator-activated receptor-α (PPARα) as a potential therapeutic target in endometrial cancer treatment. Treatment of an endometrial cancer cell line with a PPARα agonist, significantly reduced proliferation and increased cell death, suggesting that altered expression of nuclear hormone receptors involved with fatty acid metabolism may lead to deregulated cellular proliferation and apoptosis. Risinger *et al.* (2003) used cDNA microarrays to compare gene expression in endometrioid and non-endometrioid cancers to normal endometrium. Unsupervised multidimensional scaling revealed separate clusters for papillary serous, clear cell and endometrioid cancers compared with normal endometrium. This indicates consistent global gene expression changes for each cancer type. Hierarchical clustering using the genes that statistically differed between one or more of the four subgroups, showed that gene expression differences in only 24 transcripts could distinguish serous from endometriotic cancers. These arrays also revealed previously unrecognised novel pathways in endometrial cancers, such as the downregulation of SOCS2, a member of the suppressors of cytokine signalling family of intracellular proteins that are involved in the negative regulation of cytokine signal transduction.

Microarrays have been used to investigate the molecular effects of progesterone, which is known to reduce the risk of developing endometrial cancer, and PRs are found in endometrial cancer cells (Dai *et al.* 2002, Oehler *et al.* 2002, Smid-Koopman *et al.* 2003). After treatment of normal, non-transformed endometrial epithelial cells with oestradiol and the synthetic progestogen norethisterone acetate, Wnt-7a, part of the Wnt family of secreted signalling molecules, was shown to be upregulated (Oehler *et al.* 2002). Wnt signalling, therefore, may be involved in the anti-neoplastic, endometrial protective effects of progestogens.

Two studies have examined the functional difference between the two human progesterone receptor (hPR) isoforms in human endometrial cancer (Dai *et al.* 2002, Smid-Koopman *et al.* 2003). Endometrial cancer cell lines, stably transfected with either hPRA or hPRB, were treated with progestins and differential gene expression was analysed using nylon cDNA arrays. The array data revealed distinctive differences in target gene regulation between the two hPR isoforms. Only cells expressing hPRB were growth responsive to progesterone and expression levels of five different genes, insulin-like growth factor-binding protein-3, fibronectin and replication protein A (Smid- Koopman *et al.* 2003) and fibronectin, integrin $\alpha_3\beta_1$ (Dai *et al.* 2002) were down-regulated in hPRB-expressing cells. The results emphasised that the relative distribution of hPRA and hPRB in endometrial cancer cells may have great implications on the behaviour of human endometrial tumours. Down-regulation of adhesion molecules in the presence of the hPRB isoform suggests that progesterone acts principally through hPRB receptors to inhibit cancer cell invasiveness mediated by adhesion molecules (Dai *et al.* 2002).

The future of large-scale studies of endometrial gene expression

To date, microarrays have been the primary method employed in large-scale studies of gene expression in endometrium. They have been used in two main ways. The first is the comparison of tissue from normal women and patient groups. Examples include comparisons of normal and malignant tissue, or endometrium from normal women and those with endometriosis. The aim here is to identify changes in gene expression, characterising the disease state, to understand disease progression and to identify novel therapeutic targets. In cancer, careful expression profiling can distinguish different closely related tumour types and may lead to tailoring of subsequent clinical management, based on the molecular phenotype of the tumour and the likely prognosis. In future, such studies will be extended to other areas of endometrial dysfunction such as menorrhagia and unexplained infertility. Secondly, microarrays have been used to study the actions of hormones, cytokines and drugs on endometrium. Experiments to identify genes regulated by progesterone have been very successful in expanding our understanding of how this hormone or antagonists like RU486, act on endometrium. It has also led to the identification of novel therapeutic targets, for example, in the area of contraception (Alox15).

A weakness of most of these studies is their use of whole tissue samples, which means that genes expressed at low levels in a limited number of cells may not be detected. A second limitation is that if a gene is expressed in different cell types at different levels, only an average read-out is detected by microarray analysis of the whole tissue. In order to understand the responses of individual endometrial compartments, and how they may influence each other, it will be necessary to isolate individual cell types to determine their unique transcriptome. One solution to this problem is the technique of LCM, which has recently been used to study luminal gene changes induced by blastocyst apposition (Yoon *et al.* 2004). In this approach, individual cells or groups of cells such as luminal or glandular epithelium can be isolated under the microscope from tissue sections and RNA from them interrogated by microarrays. This allows a gene-expression profile specific to one cell type to be determined. An example of laser capture on murine endometrium is shown in Fig. 4. Preliminary studies using this technique have shown that it holds considerable promise to improve our understanding of the interactions between different cellular compartments in endometrium (Yanaihara *et al.* 2005). Although microarray techniques have become extremely powerful tools, many cellular functions require alterations in proteins that are not reflected in steady-state RNA levels. The development of high throughput 'proteomic' techniques is underway and these potentially offer the ability to examine effects of post-translational regulation of protein expression levels. Post-translational modification such as ubiquitination and phosphorylation also play major roles in regulating protein functions such as half-life and enzyme activity. These aspects of the cell biology of the endometrium can only be investigated by high throughput protein-based methods. A complete understanding of endometrial function will require integration of RNA data from microarray experiments with protein data from other techniques.

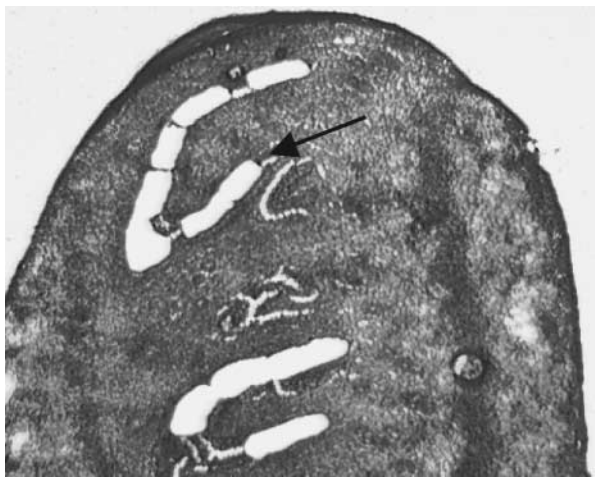

Figure 4 Laser capture microdissection of murine endometrium. Frozen sections of mouse uterus at day 3 of pregnancy were subjected to laser capture microdissection using a PALM MicroBeam system. Luminal epithelium (arrowed) was collected for RNA isolation and microaray hybridisation without stromal cells. The region from which the epithelium has been collected is clearly visible.

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