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〈Review〉

Comparative Analysis of Gene Expression in the Female Reproductive Organs

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ABSTRACT

To understand molecular and cellular mechanisms of many gene products in the female reproductive organs including the ovary and uterine endometrium as well as during embryo development, researchers have developed and utilized many effective methodologies to analyze gene expression in cells, tissues and animals over the last several decades. For example, blotting techniques have helped to understand molecular functions at DNA, RNA and protein levels, and the reverse transcription-polymerase chain reaction (RT-PCR) method has been widely used in gene expression analysis. However, some conventional methods are not sufficient to understand regulation and function of genes expressed in very complex patterns in many organs. Thus, it is required to adopt more high-throughput and reliable techniques. Here, we describe several techniques used widely recently to analyze gene expression, including annealing control based-PCR, differential display-PCR, expressed sequence tag, suppression subtractive hybridization and microarray techniques. Use of these techniques will help to analyze expression pattern of many genes from small scale to large scale and to compare expression patterns of genes in one sample to another. In this review, we described principles of these methodologies and summarized examples of comparative analysis of gene expression in female reproductive organs with help of those methodologies.

(Key words : microarray, real-time RT-PCR, suppression subtractive hybridization, differential display PCR)

INTRODUCTION

Comparative analysis of gene expression with various conventional methodologies has expanded understanding of biological function of genes in cells, tissues and organisms. Northern blot analysis has been widely used in evaluation of gene expression, which is a method to detect target mRNA using labeled cDNA or RNA probes (Alwine *et al.*, 1977). RNase-protection assay has been also used to detect expression of specific mRNA using probes hybridized with target sequences which protect target mRNA from RNases and leave transcripts of interest (Berk and Sharp, 1977). However, these approaches are available only for genes that have known sequence information to generate probes. Differential plaque-filter hybridization is another technique to identify differential expression of cloned cDNAs (Maniatis *et al.*, 1978). Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) is a very simple method to quantify levels of gene expression in cells or tissues (Gilliland *et al.*, 1990), but this method has been used for validation of other analysis data due to the limitation in analysis of gene expression in large scale and development of advanced techniques.

Techniques of differential gene expression analysis are based on either PCR or hybridization method. Real-time RT-PCR method is a sophisticated form of semi-quantitative RT-PCR and has been widely used to determine relative or absolute mRNA abundance or DNA copy number (Heid *et al.*, 1996; Livak and Schmittgen 2001; Winer *et al.*, 1999). This quantitative analysis method is developed based on 5' nuclease activity of Taq polymerase which can cleave a non-extendible hybridization probe during PCR reaction (Holland *et al.*, 1991). During PCR reaction, charge-coupled device camera detects fluorescent emission from two fluorescent dyes in real time (reporter dye and quenching or reference dye). Quenching dye

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signal is used to normalize reporter dye signal variation because there is little or no change in signal intensity of quenching dye during PCR reaction. Result is presented by C_T value which is a point amplification plot crosses threshold based on ΔRn value. Also, it is possible to screen non-specific reaction via adding a dissociation step. There are two approaches to analyze gene expression levels using the real-time PCR. One is absolute quantification to estimate copy numbers in input samples (Holland *et al.,* 1991), which presents data as international unit of weight per reference. In absolute quantification, setup of standard curve to estimate abundance of amplicons is the most critical step for accurate gene expression analysis (Heid *et al.,* 1996). The other method is to measure relative levels of expression compared to a reference gene level. In relative quantification, results are calculated by $2^{-\Delta\Delta C_T}$ method and presented as relative fold per control (Livak and Schmittgen 2001; Winer *et al.,* 1999). Thus, real-time RT-PCR is a powerful and simple method to measure gene expression levels.

However, these methodologies are applicable only for analysis of genes that have known sequence information and unsuitable for the high-throughput analysis of gene expression. To overcome the limits of those classical techniques, novel analytical methods for gene expression with high specificity and reliability have been developed and employed for the large-scale analysis of gene expression at RNA levels. Microarray, the most powerful technique, is widely used in functional genomics, and other PCR- or hybridization-based techniques also allow large scale analysis of gene expression in cells and tissues. This review focuses on the use of techniques for comparative analysis of gene expression to understand biological processes in the female reproductive organs.

METHODOLOGIES TO ANALYZE COMPARATIVE GENE EXPRESSION

1. Differential Display PCR

Differential display (DD)-PCR is a method to amplify cDNAs using oligo dT and arbitrary primer that has short sequences, in which primer pairs are considered to amplify 50 to 100 cDNAs with approximately 500 bp of product size (Liang and Pardee, 1992). This amplification enables comparison of differentially expressed genes from two or more samples. Therefore, the DD-PCR is useful in a situation with limited information and comparison of any number of samples (Stein and Liang 2002). However, DD-PCR results in high rate of false positivity and strong bias to genes that have high copy number (Bertioli *et al.,* 1995; Liang and Pardee, 1995). However, the defect of DD-PCR of high false-positive rate in identification of novel differentially expressed genes has been greatly reduced by using a class IIs restriction endonuclease and new primer design methods (Mahadeva *et al.,* 1998) compared to the conventional DD-PCR and improved reliability (Graf *et al.,* 1997). DD-PCR has significant advantages of simplicity and the possibility of detecting virtually all expressed mRNAs when using sufficient primer combinations with less labor and time. And, it is more valuable when DD-PCR is used with other techniques, such as suppression subtractive hybridization and microarray-based investigations.

In pigs, DD-PCR was firstly applied to determination of effect of basic fibroblast growth factor (bFGF) on arrested endothelial cells (Kozian and Augustin, 1995). Soon after, spermidine/spermine N1-acetyltransferase (*SSAT*), fragile X mental retardation gene (*FMR1*) and N-acetylglucosamine transfer protein of *Escherichia coli* were identified using DD-PCR technique from the peri-implantation stage endometrium in pigs (Green *et al.,* 1996). These authors demonstrated that *SSAT* expression was regulated by steroid hormone dependent manner and suggested that *SSAT* might play a important role in uterine development and differentiation during peri-implantation (Green *et al.,* 1996). On the epithelium of oviduct carrying embryo, 13 genes were identified after human chorionic gonadotrophin (hCG) injection (Chang *et al.,* 2000). They suggested that follicle stimulating hormone receptor (*FSHR*), transforming growth factor-α (*TGF-α*), transforming growth factor binding protein Ⅱ (*TGF-BP Ⅱ*) and atrial natriuretic peptide (*ANP*) may be related to 4-cell block in porcine embryos and embryo development during the transport period into the uterine lumen (Chang *et al.,* 2000). Genes that were responsible for the fetal development in the pigs were identified in uterine endometrium between 12 and 45 days of pregnancy (Wesolowski *et al.,* 2004). In ewe, Spencer and colleagues demonstrated that beta-lactoglobulin (*BLG*), alkaline phosphatase (*ALPL*), type B and D endogenous sheep retroviruses (*JSRV*), gp330/megalin (*LRP2*), matrix Gla protein (*MGP*) are originated from uterine glands (Spencer *et al.,* 1999). Nie and colleagues identified differential expression of calcium-binding protein D9k (*S100G*) mRNA during early pregnancy in mouse placenta (Nie *et al.,* 2000). Abundance of *S100G* mRNA was significantly low at implantation site than that of in inter-implantation site between day 4.5 and 5.5 with strong signal on day 4.5 of pregnancy and *S100G* mRNA was localized in luminal epithelial cells of pregnant uterus in mouse, suggesting that temporal down-regulation of *S100G* was strongly related to progesterone and may be significant for embryo attachment to uterine epithelial cells at early pregnancy in mice. Kumar *et al.* (2001) identified differentially expressed genes during the window of implantation in human using DD-PCR technique. Among those genes, they found that guanylate binding protein 1 (*GBP1*) mRNA was localized in glandular epithelial cells and stromal cells, suggesting that *GBP1* could be used as a marker of the window of implantation and be involved in mechanism of uterine receptivity (Kumar *et al.,* 2001). Using the DD-PCR technique resulted in differentially expressed genes rangedfrom tens to thousands. Thus, DD-PCR is more beneficial for identification of differentially expressed genes in small or medium scale analysis. The use of appropriate primer pairs to reduce false-positivity would make DD-PCR technique a valuable tool in reproductive biology.

2. Annealing Control Primer (ACP)-PCR

Annealing control primer (ACP)-PCR is a well-revised technique of PCR with higher specificity (Hwang *et al.,* 2003). ACP-PCR consists of two steps of which first strand cDNA is synthesized in the first step using dT-ACP1 and differentially expressed genes are subsequently amplified with the primer sets of an arbitrary ACP and dT-ACP2 (Hwang *et al.,* 2003). Arbitrary designed ACP primer enables to reduce nonspecific priming in ACP-PCR reaction. ACP primer is consisted of three distinctive sequences (Hwang *et al.,* 2003). One is core sequence of 3' end region that is complementary to template sequence. Another is universal sequence of 5' end region. The other is polydeoxyinosine [poly(dI)] linker, which mainly contributes to specificity of ACP-PCR by forming bubble-like structure at specific annealing temperature. Furthermore, poly(dI) linker prevents hybridization of 5' region with template under temperature where 3' region anneals to template. During the first round PCR, core sequence of 3' end anneals to target site of template whereas universal sequence of 5' end does not anneal to template by action of poly(dI) linker under same condition. Restricted amplification using core sequence is a key feature of ACP-PCR. During the second round PCR, primers anneal to core sequence of 3' end and universal sequence of 5' end of first round PCR product as target sequence, which prevents from further extension by 3' core sequence and increases PCR efficiency. Finally, differentially expressed gene is displayed via agarose gel electrophoresis. ACP-PCR is useful for identification of genes that are expressed at a low level and have long product sizes because of its highly annealing specificity without false positivity (Hwang *et al.,* 2004).

ACP-PCR was used to analyze expression of hatched blastocyst-specific genes in bovine (Hwang *et al.,*, 2004), which include nine differentially expressed genes, ferritin light polypeptide (*FTL*), ribosomal protein small subunit 12 (*RPS12*), lysosomal-associated protein transmembrane 4A (*LAPTM4A*), ribosomal protein large subunit 12 (*RPL12*), apoA-I binding protein (*AIBP*), CULLIN-1 (*CUL1*), high density lipoprotein (*HDLP*), cytochrome c oxidase subunit 5A (*COX5A*) and responsive to centrifugal force and shear stress gene 1 (*RECS1*), involved in zona escape of bovine blastocyst (Hwang *et al.,*, 2004). Ha and colleagues have reported that four genes including msh homeobox 2 (*MSX2*), uridine 5'-diphosphate-glucose 4-epimerase-encoding (*GAL10*), valosin-containing protein (*VCP*) and phospholipase C, eta 1 (*PLCH1*) that might be associated with regression of Mullerian ducts are differentially expressed in the Mullerian ducts of male and female chick embryos (Ha *et al.,* 2008). Recently, twelve genes have been identified in the endometrium on day 12 of the estrous cycle and pregnancy in pigs (Ka *et al.,* 2008), including FXYD domain containing ion transport regulator 3 (*FXYD3*), serum amyloid A2 (*SAA2*), swine leukocyte antigen-DQA1 (*SLA-DQA1*), crystallin, mu chain (*CRYM*), inhibitor of DNA binding 2 (*ID2*) and keratin 7 (*KRT7*), which are expressed higher levels on day 12 of estrous cycle, and creatine kinase, muscle (*CKM*), S100 calcium binding protein A7A (*S100A7A*), gelsolin (*GSN*), transient receptor potential cation channel 6 (*TRPV6*), salivary lipocalin (*SAL1*) and *KIAA1324*, which are expressed higher levels on day 12 of pregnancy. Furthermore, the results indicated that calcium-related genes, *S100A7A*, *GSN*, and *TRPV6*, may play important roles in calcium regulation during the implantation period in pigs (Ka *et al.,* 2008). ACP- PCR is an easy method to analyze comparative gene expression in a small to medium scale.

3. Serial Analysis of Gene Expression

Serial analysis of gene expression (SAGE) is a technique that enables rapid analysis, which has two marked features; one is that SAGE uses short nucleotide sequence tags that are enough to amplify specific target sequences, and the other is that concatenation of short sequence tags permits serial analysis efficiently by the sequencing of SAGE tags (Velculescu *et al.,* 1995).

In principle, cDNA synthesized using biotinylated oligo (dT) primer isdigested with restriction endonucleases with 4 bp recognition sites, and cleaved fragments are isolated by streptavidin beads subsequently. Anchoring enzyme synthesizes complementary sequence and generates blunt ends. Then, cDNA fragment is divided in half and ligated with one of two linkers containing a type IIs restriction site wherein 20 bp away from type IIs endonucleases (tagging enzyme) recognition site is cleaved. Anchoring enzyme and tagging enzyme produces a short tag of 9 bp. Two pools of tags are ligated to each other, which serves as templates for PCR with linker specific primers. This step provides for orientation and punctuation of tag sequence and a means to completely exclude potential distortions by PCR. And, cleavage of PCR product with the anchoring enzyme enables separation of ditags which is ligated, cloned, sequenced and analyzed. Therefore, the SAGE technique requires well-organized database for global analysis of transcription profiles as like mouse and human systems (Stollberg *et al.*, 2000). For this reason, SAGE has been less used in global analysis of gene expression of farm animals. Accomplishment of genome projects in farm animals would give more chances to determine gene expression of farm animals in a small or medium scale. This SAGE technique is relatively fast, cost-effective, and suitable for identification of cell or tissue specific gene expression (Lievens *et al.*, 2001; Stollberg *et al.*, 2000). However, SAGE analysis results in underestimation of the copy number of actively expressed genes or rarely expressed genes, and false-positive unique SAGE tags due to sequencing error (Stollberg *et al.*, 2000).

Blomberg *et al.*, (Blomberg *et al.*, 2005) firstly applied SAGE technique to identify specific gene expression in developing porcine embryos just prior to attachment. They reported that 431 SAGE tags were expressed differentially between ovoid and filamentous conceptuses participating in morphogenesis of developing conceptuses between day 11 and 12 of pregnancy and determined genes associated with steroidogenesis, such as cytochrome P-450(scc) (*CYP11A1*), aromatase (*CYP19A*), and steroidogenic acute regulatory protein (StAR), and oxidative stress response-related genes, such as microsomal glutathione S-transferase 1 (*MGST1*) and copper-zinc superoxide dismutase (*SOD1*) (Blomberg *et al.*, 2005). Mihm and coworkers (2008) compared gene expression in granulosa cells of dominant follicles and growing cohort follicles using the SAGE technique and demonstrated that 8 of 93 transcripts, such as cyclin D2 (*CCND2*), growth arrest and DNA damage-inducible beta (*GADD45B*), splicing factor arginine/serine rich 9 (*SFRS9*) and ovary-specific acidic protein (*DQ004742*) were differentially expressed in granulosa cells isolated from dominant follicles. Ma and coworkers (Ma *et al.*, 2006) investigated differential gene expression of the implantation and inter-implantation sites in the mouse uterus and found approximately 100,000 of SAGE tags. They determined 100 and 127 tags that were significantly up-regulated by site-specific manner at implantation and inter-implantation site respectively.

4. Suppression Subtractive Hybridization

Suppression subtractive hybridization (SHH) is a combined method of subtractive cDNA hybridization and representational difference analysis (Diatchenko *et al.*, 1996). SHH is a PCR-based cDNA subtraction method to identify differentially expressed genes by suppressing undesirable amplification. cDNA subtraction method identifies differentially expressed genes between two samples by physical seperation of unhybridized fragment after hybridization of cDNA from one population (tester) to excess of cDNA from other population (driver) (Hara *et al.*, 1991; Hedrick *et al.*, 1984). Driver population is prepared by restriction enzymes digestion of cDNAs and tester population is prepared by ligation of two different adaptor sequences to cDNAs digested with restriction enzymes. Four forms of fragments including single stranded (ss) cDNA tester fraction, homo-hybrid cDNAs, hetero-hybrid (common non-target cDNA) and non-hybridized ss cDNA could be generated by adding driver to testers in the first hybridization. In the second hybridization, additional driver was added to testers, which was mixed and hybridized again. Subtractive and normalized fraction could be newly generated in the second hybridization step, which involved two adaptor sequences in its 5' and 3' end. Subsequent PCR isa step to amplify subtractive and normalized fraction. SSH is suitable for the isolation of differentially expressed genes with high and/or low abundance and is reliable because the SSH procedure includes normalization step in the subtraction procedure (Diatchenko *et al.*, 1996). SHH method enables to identify differentially expressed genes with less time and effort. However, it has a disadvantage of inefficiency for low abundance transcripts (Tuggle *et al.*, 2007). And it requires more amount of mRNA than used in other technique.

Using this method, 142 genes that were differentially expressed in elongating conceptuses, from spherical, tubular to filamentous conceptuses, were evaluated their contribution to morphogenesis (Ross *et al.*, 2003). Among them, abundance of ribosomal RNAs decreased whereas interleukin 1-β (*IL1B*), thymosin beta 4 (*TMSB4*), mitochondrial cytochrome B (*MTCYB*), heat shock cognate (*HSC70*) and S-adenosyl homocysteine hydrolase (*SAHH*) increased as elongation proceeded (Ross *et al.*, 2003). Especially, expression of *HSC70* and *SAHH* was significantly expressed during the period of embryo transition, which might play a significant role in conceptus elongation (Ross *et al.*, 2003). Vallee and co-authors (Vallee *et al.*, 2003) also used SHH method to identify differentially expressed genes in Meishan-Landrace conceptuses and endometrial tissue at day 15 of pregnancy. They determined 137 endometrial- and 166 conceptus-enriched cDNAs and evaluated 20 genes that were related to embryo survival. Wu and colleagues (Wu *et al.*, 1999) examined expression of extracellular matrix protein expression in the myometrium of pregnant ewes in spontaneous term labor and in betamethasone-induced premature labor, showing that thrombospondin-1 (*TSP1*) may be crucial for myometrium contraction during parturition in ewes, and further analysis revealed that *TSP1* was mainly produced from myometrial fibroblasts and the smooth muscle cells (Wu *et al.*, 1999).

5. Expressed Sequence Tag

Initially, expressed sequence tag (EST) analysis has been designed for identification of mRNA coding sequences by assembling overlapping EST with primer sets (Adams *et al.*, 1991) or physical mapping (Jones *et al.*, 1995). EST technique is a simple, but labor-intensive technique because it needs to construction of EST libraryby unidirectional cDNA synthesis and sequencing that allows comparison of EST expression from two or more cells or tissues. In pigs, it has been reported that 2,489 clusters are found in germinal vesicle stage oocytes, 4-cell embryos and blastocysts, showing embryo stage-specific EST contigs (Whitworth *et al.*, 2004). As cDNA library database is constructed using porcine tissues (Davoli *et al.*, 2002; Nobis *et al.*, 2003; Tuggle *et al.*, 2003; Yao *et al.*, 2002), EST technique has been more useful in both differential gene expression study and physical mapping or characterization of coding sequence in farm animals. Recently, ESTs have been used for detection of single nucleotide polymorphism (SNP) in placenta by collecting and comparing EST contigs from various tissue and individuals in human (Hayes *et al.*, 2007). Although EST technique requires more labors and times than DD-PCR and ACP-PCR techniques, it is suitable for medium scale analysis and could provide more information about reproduction of farm animals.

6. Microarray Analysis

Microarray analysis technique has been widely used in large scale gene expression studies of various systems since it was developed. Initial concept of array was proposed by Kulesh and colleagues in 1987 (Kulesh *et al.*, 1987). They prepared cDNA libraries from IFN-treated or -untreated human fibrosarcoma hybridized with proliferating and quiescent associated probes (Kulesh *et al.*, 1987). More modern concept of microarray was developed by Schena *et al.* (1995), who used robotics to print cDNAs on glass and two-color fluorescence hybridization and identified 45 of differentially expressed gene in Arabidopsis. Development of commercial microarray chip evokes identification of gene expression profiles in various systems (Chee *et al.*, 1996). As more powerful and reliable platforms have been developed, microarray analysis has been widely used in various systems to analyze gene expression profile in a large scale. Microarray technique is the assembly of RNA isolation and purification, cDNA synthesis and nucleic acid labeling for hybridization, and bioinformatics for data analysis. Platform for microarray analysis is a microscope slide with dimensions of 25 × 75 mm and probes that are representing unique gene respectively. Synthetic oligonucleotides and cDNAs are used in microarray analysis to measure gene expression levels. Total RNAs or mRNAs from control and test sample are hybridized with fluorescent dye of Cy5 and Cy3. After hybridization, the fluorescent signal of the hybridized probes is detected with laser scanner and arbitrary intensity of probe hybridization are normalized and analyzed with bioinformatic algorithms and database.

Caetano and colleagues' work is one of early reports using cDNA microarray analysis technique for detection of differential expression profiles in ovary (Caetano *et al.*, 2004). They compared ovarian gene expression between lines ovulating 6.7 ova and random selected commercial lines using cDNA microarray chips spotted 4,600 probes and determined genes related to ovarian capacity and reproductive performances (Caetano *et al.*, 2004). Gladney and colleagues analyzed gene expression profiles in ovarian follicles of selected lines based on index of ovulation rate and embryo survival, and control lines using two microarray platforms (Gladney *et al.*, 2004). Also, gene expression profiles during folliculogenesis and luteinization were determined in pigs (Agca *et al.*, 2006; Zielak *et al.*, 2008; Zielak *et al.*, 2007) and in bovine using microarray technique (Mihm *et al.*, 2006).

Whitworth and co-workers determined gene expression profiles between vesicular oocytes, four-cell, blastocyst, *in vitro*-produced four-cell, and *in vitro*-produced blastocyst stage embryos to identify key genes, such as destrin (*DSTN*), poly(A) binding protein interacting protein 1 (*PAIP1*), ubiquitination factor E4B (*UBE4B*), nuclear autoantigenic sperm protein (*NASP*), high-mobility group box 1 (*HMGB1*), ATP synthase, H^+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1 (*ATP5A1*), U6 SnRNA-associated Sm-like Protein (*LSM2*), DnaJ (Hsp40) homolog, subfamily B, member 6 (*DNAJB6*), and activated leukocyte cell adhesion molecule (*ALCAM*), during embryogenesis in pigs (Whitworth *et al.,* 2005). Lee *et al.* (2005) analyzed gene expression profile from elongating conceptuses including small spherical, large spherical, tubular and filamentous stage in pigs using cDNA microarray (Lee *et al.,* 2005). And, they suggested that 9 genes, such as *STAR*, transforming growth factor-β3 (TGFβ3), *IL1B* and TMSB4 and novel transcripts could be closely related to trophoblatic elongation leading to successful attachment into endometrium. Microarray analysis technique also used in elucidating embryonic genome activation (Misirlioglu *et al.,* 2006), developmental efficiency (Assidi *et al.,* 2008) and evolutionary conserved gene expression (Vallee *et al.,* 2006). These findings may be helpful for understanding of embryonic genome activation and early development of bovine embryos.

Gene expression profile in porcine uterus during estrous cycle was also determined using cDNA microarray (Green *et al.,* 2006). They defined gene expression profiles on cyclic endometrium of day 0, 3, 6, 9, 12, 15 and 18. Total 4,827 differentially expressed genes on endometrium during estrous cycle were identified and categorized into 6 groups by *k*-mean clustering analysis. Six functional categories may seem to be closely related to uterine events in pigs, such as sperm maturation, blastocyst growth and position and conceptus development and attachment. These results provided further insights on biological function of cyclic uterus of pigs. Effects of estrogen on porcine endometrium were evaluated by treatment of estrogen cypionate into uterus of pregnant gilts using oligonucleotide microarray (Ross *et al.,* 2003). They identified 8, 32 and 5 of up-regulated and 1, 39 and 16 of down-regulated genes on day 10, 13, and 15 of endometrium of estrogen cypionate treated gilts respectively and suggested that aberrant expression of aldose reductase (*AKR1B1*), secreted phosphoprotein 1 (*SPP1*), CD24 antigen (*CD24*), and neuromedin B (*NMB*) by pre-treatment of estrogen cypionate might be related to attachment failure and degeneration of conceptuses (Ross *et al.,* 2003). Ka *et al.* recently reported that gene expression profiling in endometrium carrying somatic cell nuclear transferred (SCNT) embryos using the Platinum Pig 13K oligonucleotide microarrays (Ka *et al.,* 2007). As a result, expression of 351 genes significantly increased or decreased in the uterine tissues with SCNT embryos compared to those with normal embryos. Aberrant expression of genes related to steroidogenesis and extracellular matrix remodeling and secretory proteins might be one explanation for abnormal fetal development (Ka *et al.,* 2007).

Gene expression profile was also achieved in cyclic endometrium of bovine using customed cDNA microarray more recently (Mitko *et al.,* 2008). They collected endometrial tissues of day days 0 (oestrus), 3.5 (metoestrus), 12 (dioestrus) and 18 and identified 269 of differentially expressed genes during estrous cycle. And, they determined two distinct gene expression profiles of highest on oestrous phase and on luteal phase. The most distinctive feature of this study is a putative gene expression network models involved in endometrial remodeling, regulation of angiogenesis, regulation of invasive growth, cell adhesion and embryo feeding. These finding elucidated important molecular events in bovine endometrium during estrous cycle and would give further insights to investigate bovine endometrium. Interferon τ (INFT) regulated genes were examined in human 2fTGH and U3A (STAT1 null 2fTGH) cell lines using Affymetrix human genome U95Av2 microarray (Kim *et al.,* 2003). INFT has been well-known for pregnancy recognition signal in sheep. INFT signal mediated via signal transducer and activator of transcription 1 (STAT1)-dependent pathways. They found 101 of interferon regulated genes in 2fTGH cells and 66 differentially expressed genes in U3A cells. And, they also determined tissue-specific action and induction of INFT-related genes, which may be important establishment of pregnancy of sheep.

Microarray has also been used for investigation on differential gene expression at implantation sites and hormonal action during the window of implantation in mouse and human. Chen *et al.* reported that genes participated in embryo apposition, adhesion and tissue remodeling were differentially expressed at implantation site compared to that in inter-implantation sites in mouse (Chen *et al.,* 2006). Popovici and co-authors determined gene expression profiles that were related to decidualization of human stromal cells after stimulation by progesterone and cyclic AMP (Popovici *et al.,* 2000). Similarly, Brar and others defined 170 genes as decidualization related genes among

6,918 of differentially regulated genes in human endometrial fibroblasts during decidualization (Brar *et al.,* 2001).

Hormonal status during the window of implantation in human has been well elucidated. Kao *et al.* have provided further insights on gene expression during the window of implantation period in human (Kao *et al.,* 2002). Progesterone induced gene expression in stromal cells was additionally reported by Okada and colleagues (Okada *et al.,* 2003), which revealed that genes for immune modulators, DNA/chromatin-related proteins, signal transduction, transcription factors, transport proteins, enzyme, receptor and structural proteins were repressed by progesterone. Additionally, Punyadeera *et al.* (2005) identified novel estrogen responsive genes and progesterone regulated genes (Punyadeera *et al.,* 2005). Direct binding of estrogen to estrogen response element has been investigated in mouse epithelial cells, which has revealed that estrogen exerts its proliferative effects on uterine epithelial cells of mouse via estrogen independent pathway (O'Brien *et al.,* 2006). Tierney and co-workers investigated protein kinase C pathway after treatment of cAMP analogue in human endometrial stromal cells (Tierney *et al.,* 2003). And, they determined temporal gene expression pattern by cAMP treatment in the human endometrial stromal cells (Tierney *et al.,* 2003). Especially, Gielen and others suggested that estrogen promoted cell proliferation via activation of AKT pathway and growth factor receptor signaling (Gielen *et al.,* 2007). Lobo *et al.* (2004) found that immune related genes including decay accelerating factor (*DAF*), indoleamine 2,3 dioxygenase (*IDO*), interleukin-15 (*IL-15*), IL-15 receptor alpha subunit (*IL-15RA*), interferon regulatory factor-1 (*IRF-1*), lymphotactin (*Lpn*), natural killer- associated transcript 2 (*NKAT2*) and granulysin (*NKG5*) were up-regulated during the window of implantation in human (Lobo *et al.,* 2004). It has been reported that *IL1B*, a important regulator in pregnancy, may be participated in free radical protection and fatty acid metabolism by regulating related genes in human endometrial cells (Rossi *et al.,* 2005).

CONCLUSION

Classical differential gene expression analysis techniques have been very useful for investigation of genomes and transcriptomes. For the limitation in large scale analysis, classical differential gene expression analysis techniques have been replaced with revised or newly developed techniques as mentioned above. PCR-based techniques are suitable for the analysis of gene expression from small scale to medium scale for the limited combination of primer pairs. These techniques have advantages of cost- and time-effectiveness, but cause high false-positivity. Hybridization-based techniques are useful in determination of gene expression profiles from medium to large scale. In addition, microarray analysis enables to estimating biological mechanisms and characteristics in given conditions by construction of putative gene expression network.

However, there are some problems to solve in application of these differential analysis techniques. First, more reliable primer pairs are required to reduce false-positive rate in use of PCR-based technique. Primer pairs used in DD-PCR and ACP-PCR have characteristics of lower specificity and higher sensitivity for templates to obtain more differentially expressed genes as many as possible than general primer pairs used in PCR. Comparatively short primer length may also be factors to increase false-positive rate. Therefore, optimal primer design considering specificity, sensitivity and length would improve performance of differential analysis technique. Second, reproducibility of microarray hybridization is faithful for identification of differentially expressed genes if there were little or no variation between RNA samples in a same laboratory. However, it has been shown that reduced concordance and increased noise have been found in among laboratories. Although functional analysis of gene expression is now available via computational methods, it is hard to obtain meaningful data from functional analysis of gene expression for the redundancy of gene function and dynamic regulation. Finally, all mRNAs are not translated into protein and the abundance of mRNA is not always consistent with the amount of protein. For this reason, it is required to analyze biological and molecular events at the protein level, which would provide precise understanding molecular mechanism in female reproductive tracts. This could be achieved with the help of using a proteomics approach, which is now being available.

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