Applications of Microarray in Reproductive Medicine

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In the genomic era, cDNA microarray (DNA chip) technology has become a very important and powerful tool for high-throughput comprehensive analysis of gene expression, genotyping and re-sequencing applications in almost every field of biomedical research. Large-scale transcriptional profiling analyses using microarrays are frequently used to explore gene expression patterns in order to better understand the molecular mechanisms of physiology and pathogenesis: these are the basis for the development of new diagnostic and therapeutic strategies. Recently, this powerful technique has been used with laser capture microdissection (LCM) and linear aRNA amplification (in vitro transcription), and used in reproductive medical research. Recent studies of microarray-related techniques used to study oocyte fertilization, early embryo development, implantation and some infertility-related diseases (endometriosis and myoma) are discussed and summarized in this review. *(Chang Gung Med J 2006;29:15-24)*

Key words: microarray, embryo, endometriosis, myoma.

Introduction

Now that the human genome project has been completed, the life scientists are now undertaking a general and integrated study of gene expressions and the functional elucidation of these genes and proteins. For this reason, various new techniques have been developed, and DNA microarray technology is the most representative one. Microarrays can simultaneously measure the expression of thousands of mRNAs. This high-throughput technique can be used to predict the function of unknown genes in medical diagnostics and biomarker discovery, to infer networks from the regulatory interactions between genes, and to investigate the mechanisms by which a drug, disease, mutation and environmental condition affects gene expression and cell function. Since the microarray technique was developed in mid-1990,[1] this high-throughput tool has been the most powerful tool for answering physiological or patho-physiological questions.[2]

An early development of microarray clinical applications was profiling differential gene expression patterns of benign and malignant tissues. These tumor-related expression profiles have been identified and are considered tumor-specific markers in prostate, breast, ovarian and endometrial cancers.[3-8] The information gathered using microarrays could be very helpful in diagnosis, prognosis, molecular classification of diseases and new drug development. However, it is only in recent years that the application of microarray technology has extended beyond the limited examination of malignant tissue.

After 2000, the microarray technique was applied to the field of reproductive medicine, including early embryo development, hormone regulation, endometrial receptivity, placental development and some gynecological diseases, including endometriosis and leiomyoma. Most of these microarray-based studies were focused on the gene expression profiles, differential expressed gene patterns in different development stages or between normal and pathological tissues. These studies are summarized and

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Received: Nov. 6, 2005; Accepted: Dec. 8, 2005
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discussed in this article.

Microarray in early embryo development

Early embryo development is a very complex process that consists of a sequential maturation of oocyte (folliculogenesis and oogenesis) and sperm (spermatogenesis), fertilization and early pre-implantation embryo development (2-cell, 4-cell, morula, blastocyst and hatching). Obviously, many may consider microarray techniques to be very helpful for studying this sequential process. In 2001, instead of traditional Reverse Transcription-Polymerase Chain Reaction (RT-PCR), this technique was first used to study the gene expression in folliculogenesis.[9] Of 588 known studied genes, the results showed that 15 detected genes were down-regulated and 46 were up-regulated as the follicles advanced to mature stages (preantral vs Graafian follicles). However, the application of microarray in early embryo development was still limited.

The major difficulty for experimentation was the limited availability and low quality of cells or RNA. Samples from early developed embryos are usually too small to yield enough RNA for direct use in microarray experiments. This problem has been solved by the in vitro RNA amplification technique (T7 RNA polymerase based in vitro linear RNA amplification), which has been reported as a reliable, reproducible and linear RNA amplifying technique and has successfully been used in microarray.[2,10] Several studies combined these two techniques to study the stage-specific genes or gene expression profiles during early embryo development. These studies show that several genes are dynamically changed in each step of zygote maturation (spermatogenesis, oogenesis and folliculogenesis) and pre-implantation embryo development.

The differential expressed genes from human oocyte, primary oocyte, secondary oocyte, to day 3 embryo (8-cell) were profiled and clustered.[11,12] Following amplification and hybridization to 40,000 targets, 1,896 of them demonstrated significant changes in expression following fertilization through day 3 of development.[12] Comparing the expression profiles of metaphase II oocytes collected from 5- to 6-week-old mice with those collected from 42- to 45-week-old mice, several age-related genes were identified in mice oocyte using the NIA 22K 60-mer oligo microarray.[13] These aged oocyte specific genes were involved in mitochondrial function and oxidative stress, chromatin structure, DNA methylation, genome stability and RNA helicases, suggesting their role involves additional mechanisms for aging. These results have implications for aging research as well as for clinical ooplasmic donation to rejuvenate aging oocytes.[13]

Pre-implantation embryo development attracted much interest while the RNA amplification and microarray techniques were being developed. The gene expression profiles from oocyte to blastocyst have been identified in mice.[13] Dr. Hamatani's study group found that global expression profiles of trophoblast development were characterized by the distinctive patterns of maternal RNA degradation and zygotic gene activation, including two major transient waves of de novo transcription. The first wave corresponds to zygotic genome activation (ZGA); the second wave, named as mid-preimplantation gene activation (MGA), precedes the dynamic morphological and functional changes from the morula to blastocyst stage. The hatching stage specific expressed gene profile has also been identified in mice by our group. However, the gene expression profiles of the human pre-implantation embryo development process have only been identified from oocyte to 8-cell embryo (day 3 embryo) and some data from "*in vitro*" trophoblast differentiation-related transcriptomic profiles.[12,13] These transcriptomic data might be helpful for developing new strategies for preimplantation genetic diagnosis (PGD) using microarray (DNA chip).

Microarray in endometrial receptivity and placental development

Endometrial receptivity is another very popular topic for the microarray approach. Successful implantation is the result of reciprocal interactions between the implantation-competent blastocyst and receptive uterus. The endometrium undergoes cyclic changes in response to circulating ovarian steroid hormones as it prepares for implantation. This dynamic tissue is well suited to microarray expression profiling for elucidation of molecular players participating in the maturation of the endometrium and during the process of implantation, i.e. "the implantation window". Previous studies have identified the dynamic gene expression changes in endometrial samples obtained from the same patient

two and seven days after the luteinizing hormone (LH) surge (day LH + 2 and day LH + 7),[14-16] or on day 16 (LH + 3) or on day 21 (LH + 8).[17] Semiquantitative RT-PCR confirmed that the endometrium from natural cycles in the pre-receptive phase (LH + 3, day 16) expressed higher levels of phosphatidylinositol-4-phosphate 5-kinase type I beta (PP5K1B) and msh homeobox homolog 1 (MSX1), whereas annexin IV (ANXA4), secreted phosphoprotein 1 (SSP1 or osteopontin) and Forkhead box O1A (FOXO1A) were expressed in higher levels in the receptive (LH + 8, day 21) endometrium.[17] Microarray data from five previously published studies indicated that SPP1 was up-regulated during the putative window of implantation in all studies; CD55, monoamine oxidase A (MAOA), IL-15 and MAP3K5 were found in at least four studies.[8,17-20] The role of these genes and their products could lead to new approaches for improving the management of early recurrent pregnancy loss, optimizing embryo implantation in IVF therapy, and could also be the basis for the development of new contraceptive strategies.

Decidualization is critical for embryo implantation and placental development. Dr. Brar has characterized gene expression pattern kinetics during decidual fibroblast differentiation by microarray analysis. Of 6,918 genes analyzed, 121 genes were induced by more than twofold, whereas 110 were down-regulated, including the insulin receptor, some neurotransmitter receptors, neuromodulators, follicle-stimulating hormone receptor, inhibin/activin-βA subunit, inhibin-α, and tumor necrosis growth factor-related apoptosis-inducing ligand (TRAIL).[21] Decidual and villous placental tissues obtained from first trimester abortus were also analyzed via cDNA microarray in our previous study,[22] which provided insight into the interactions between human trophoblastic cells and deciduas in early placental development. We found 21 genes that increased > 10-fold in villi compared with decidua, 10 were hormone-associated genes including LIF-R, chorionic gonadotrophin-β, PP5 (placental protein 5), placental lactogen (chorionic somatomammotrophin hormone 1), inhibin βA, and the SP-1 family (pregnancy-specific β1 glycoprotein). These results may help us understand trophoblast differentiation, decidual gene regulation and placental development, and may also provide new information for genetic diagnosis or gene therapy of some abnormal placenta and abortion in preterm birth. The microarray-based studies in early embryo development and endometrial development before and after implantation are summarized in Table 1. Global profiling of genes in embryo development, endometrium, decidua and at the interface between the trophoblast and the decidua has provided remarkable insights into endometrial maturation and implantation.

Microarray in endometriosis

Endometriosis is a benign gynecological disorder associated with pelvic pain and infertility, with the latter being due in part to bad oocyte quality and failure of embryonic implantation in the maternal endometrium. There are many microarray based studies that were focused on this topic (Table 2). The first paper regarding discovery of the endometriosis markers using cDNA microarray was published in 2002.[23] Only eight genes from a total of 4,133 genes on the DNA microarray increased in endometriosis implants compared with uterine endometrium. The following studies have identified more endometriosis-related genes through a larger scale (or genome-wide) cDNA microarray platform in ovarian endometrial cysts.[24,25] The up-regulated genes encoded some HLA antigens, complement factors, ribosomal proteins and TGFBI, whereas the down-regulated elements included the tumor suppressor TP53, genes related to apoptosis such as GADD34, GADD45A, GADD45B and PIG11, and OVGP1.[25] According to our previous cDNA microarray analysis, we have identified 92 genes whose expression was specifically higher in eutopic endometrium with endometriosis (as compared with a normal group), and also expressed higher in eutopic endometriotic tissues without GnRHa treatment, including glutathione S-transferase M4, integrin beta 4 binding protein, retinoblastoma-binding protein 4 and fatty-acid-Coenzyme A ligase. The genes related to cell growth (PCNA, CDC2 delta T and topoisomerase II alpha), cell transformation (pituitary tumor transforming factor) and cell invasion (H-cadherin, VCAM-1, cathepsin B, Enolase 1 alpha) were also highly expressed in the ectopic endometric tissues without GnRHa treatment compared to GnRHa treated ectopic or eutopic endometrial groups.[24,26] The differential gene expression profiles in ectopic endometrium (chocolate cyst) with or without

Table 1. Microarray Studies in Early Embryo Development and Implantation

Topics and samples	Species	References
Folliculogenesis (preantral vs Graafian follicles)	Human	9
Newborn compared to adult ovary	Mouse	45
Spermatogenesis	Human, Mouse	46
Oocyte	Human	11
Oocyte-specific genes	Bovine, Mouse, Xenopus	47
Aged oocyte (5~6 *vs* 42~45 week-old)	Mouse	48
Primary oocyte, secondary oocyte in metaphase I, fertilization-competent secondary oocyte in metaphase II, 1-cell embryo, 4-cell, and 8-cell embryo.	Human	12
Preimplantation embryo development (unfertilized, fertilized oocyte, 2-cell, 4-cell, 8-cell, morula, blastocyst).	Mouse	49
Morula versus blastocyst	Mouse	54
Blastocyst dormancy and activation	Mouse	50
Blastocyst hatching	Mouse	51
Oocytes, zygote, 2-, 4-, 8-cell embryo, compacting embryo, morula and blastocyst "*in vitro*" *vs* "*in vivo*".	Mouse	52
Trophoblast differentiation	Human	13
Endometrial receptivity	Human	14-20
(LH+2-4 vs LH+7-9)		
(peak E2; cycle d 8-10 vs LH+8 -10)		
(peak E2; cycle d 9-11 vs LH+6 -8)		
(LH+2 vs LH+7)		
(LH+2 vs LH+7)		
(LH+2 vs LH+7)		
(LH+3 vs LH+8)		
Endometrial fibroblast decidualization "*in vitro*"	Human	21
Endometrial stromal cells decidualization "*in vitro*"	Human	53
Early gestational decidua and chorionic villi	Human	22
Mid-gestation placenta and embryo	Mouse	55

Gonadotropin-releasing hormone (GnRH) analog treatment were also examined using human cDNA microarray.[27] The effects of GnRHa on gene expression profiles were also studied on a murine gonadotrope tumor cell line (LbetaT2).[28]

Using a laser capture microdissection (LCM) technique, the differentially expressed genes were investigated in epithelial and stromal cells from deep endometriosis and matched eutopic endometrium via cDNA microarrays.[29] The data showed that platelet-derived growth factor receptor alpha (PDGFRA), protein kinase C beta1 (PKC beta1) and janus kinase 1 (JAK1) were upregulated, and Sprouty 2 and mitogen-activated protein kinase kinase 7 (MKK7) were down-regulated in endometriosis stromal cells, suggesting the involvement of the RAS/RAF/MAPK signaling pathway through PDGFRA in endometriosis pathophysiology.[29] The same group further investigated mRNA expression of RON (a tyrosine kinase receptor) and its ligand, macrophage stimulating protein (MSP) in deep endometriotic lesions to confirm the previous microarray data by using LCM and quantitative real-time RT-PCR. According to their findings, the MSP/RON system may be involved in the pathophysiology of endometriosis.[30] A better understanding of the biochemical mechanisms underlying gene expression of the normal endometrial cycle and the abnormalities in endometriosis will facilitate development of new diagnostic criteria beyond histological evaluation, and will permit identification and validation of molecular targets for future drug discovery.[31] However, when the results from these studies were compared, no common genes or pathways could be identified as markers for endometriosis progression. Ways to reduce individual differences between patients and better studies are very important for this type of approach in the future.

Table 2. Microarray Studies in Endometriosis and Leiomyoma.

Topics/Samples	Phase/Treatment	Array Platform	References
Endometriosis			
Cyst wall of the endometrioma vs uterine endometrial biopsy (n = 3)	Follicular phase	[^{33}P]-labelled cDNA; Named Human Genes GeneFilters (Research Genetics, Huntsville, AL). (4,133 named human genes)	23
Ovarian endometrial cysts vs eutopic endometria (n = 23)	Proliferative and secretory phase	cDNA microarray consisting of 23,040 genes	25
Cyst wall of the endometrioma vs uterine endometrial biopsy (n = 3)	GnRHa treated	cDNA microarray consisting of 9,600 genes	24,26,27
Deep endometriosis (rectovaginal nodules) (n = 6)	Proliferative and secretory phase	LCM+T_7 based linear amplification; [^{32}P]dCTP labeled cDNA, Clontech Atlas™ human 1.2 cDNA expression array (1,176 genes)	29
Human ovarian surface epithelium (HOSE) cells	(IL-1α treated)	cDNA microarray consisting 256 genes	56
Eutopic endometrium (n = 9), ovarian endometriomas (n = 26), and peritoneal endometriotic lesions (n = 12)	Proliferative and secretory phase	2-mm Tissue array	57
Normal and ectopic endometrium	RU486-expose endometrium	oligonucleotide gene array	58
Normal and ectopic endometrium	-	cDNA microarray	59
Leiomyoma			
Leiomyoma vs control myometrium (n = 9)	Follicular and luteal phases	GeneChip HuGeneFL6800 and U95A arrays (Affymetrix) (6,800 genes)	60,61
Fibroid and normal myometrium (n = 28)	-	10,500 human cDNA microarray (www.CCGPM.org)	34
Leiomyoma and surrounding myometrium (n = 7)	Proliferative phase	HuGeneFl6800 arrays (Affymetrix) (6,800 genes)	38
Fibroid and myometrial tissue (n = 5)	-	U-133 Affymetrix chip (33,000 genes)	62
Leiomyoma and myometrium (n = 6)	GnRHa treated	Clontech Atlas 1.2K cancer microarray (1,176 known genes)	42
Leiomyoma and myometrium (n = 6)	-	17 k human microarrays+2-D SDSPAGE/MS	63
Leiomyoma and matched myometrium (n = 6)	GnRHa therapy	human U95A Affymetrix GeneChip arrays	44
Cells isolated from leiomyoma and myometrial	TGF-β treated	human U95A Affymetrix GeneChip arrays	43
Leiomyoma and myometrium (n = 10)	Proliferative phase	DNA chip (Genetrack Human 7.5K cDNA chip; Genomictree Products	64

Microarray in leiomyoma

Uterine leiomyomata are benign, monoclonal, smooth muscle cell tumors that are found in up to 70% of reproductive age women.[32] Leiomyomata represent a significant public health problem because of their prevalence and associated problems of pelvic pressure, uterine bleeding, pain and infertility. Myoma fibroids have a reduced microvascular density when compared with adjacent myometrial tissue.[33] Using cDNA microarray, 25 genes with differential gene expression between fibroid and myometrium were identified.[34] Insulin-like growth factor-2, endothelin A receptor, connective tissue growth factor (CTGF), cysteine-rich angiogenic inducer 61 (CYR61) and collagen 4alpha2 (COL4A2) were confirmed by RT-PCR. CTGF and CYR61, both angiogenesis promoters, were reduced in expression relative to myometrium. COL4A2, the precursor for the angiogenesis inhibitor canstatin, was increased relative to myometrium. These three genes display an anti-angiogenic expression profile in fibroids relative to myometrium. These findings may explain the reduced microvascular density seen in fibroids relative to myometrium.

Comparing leiomyoma and normal myometrium tissues, several interesting genes were highly up-regulated, including phosphatidylinositol 3-kinase (PI3K), doublecortin, JM27, insulin receptor sub-

trate-1, ionotropic glutamate receptor subunit 2, apolipoprotein E3, IGF2, semaphorin F, myelin proteolipid protein, frizzled, CRABP II, stromelysin-3 and TGFβ3.[35] The up-regulation of PI3K and insulin receptor subtrate-1 is most interesting, and seems to interconnect with pathways that have expanded the traditional (nuclear) mechanism of estrogen action.[36] Transcription factors such as c-fos, ATF3, orphan receptor TR3 or Nur77, and glucocorticoid receptors α and β were reduced in leiomyomata, as were several proteins involved in cell-cell contact and regulation of cytoskeletal rearrangement (ARHGEF6, GTPase guanylate-binding protein 2, cadherin-13, MAP kinase kinase kinase 5, tenascin-X, extracellular-matrix protein 2).[35] Additionally, Dr. Li[37] used Affymetrix arrays HuGeneFL6800 and found that the following genes were up-regulated in leiomyomata: IGF2, IGFBP5, β-glycoprotein-11, TIMP-3, EGR-2, p53 and others.[37] In Dr. Wang's study, mRNA levels of 6,800 genes were simultaneously measured in proliferative phase uterine leiomyomata tissue and in adjacent normal myometrium using HuGeneFL® (Affymetrix, Inc., Santa Clara, CA) probe arrays. Twenty-three genes showed increased expression and 45 showed decreased expression in leiomyomata compared with normal myometrium.[38] Using UniGEM V cDNA microarray (Incyte Genomics Inc.), neuron-specific protein PEP-19 (Purkinje cell protein 4, PCP 4) was shown to be strikingly different in expression between leiomyoma and myometrium, suggesting that PEP-19 might be involved in leiomyoma pathogenesis.[39]

It is clear that leiomyoma growth is estrogen-dependent and progesterone-related since tumors regress after treatment with GnRH agonists, which inhibit leiomyoma aromatase and induce a hypoestrogenic state.[40] Microarray analysis allowed us to identify the profile expression of several specific genes in leiomyoma and myometrium, expressions which appear to be differentially regulated after GnRHa therapy. Several novel genes or new pathways could be significantly regulated by GnRHa in leiomyoma treated with GnRHa and/or TGF-β through microarray analysis.[41-43] EGR1, a prototype of a family of zinc finger transcription factors that includes EGR2, EGR3, EGR4 and NGFI-B, has been reported differentially expressed in leiomyoma and myometrium, and regulated by GnRHa therapy in leiomyoma and myometrium.[44] In our study, we have identified that expression of 172 genes was higher in myoma without treatment (e.g. Stromal cell-derived factor 1, SDF-1), and expression of 70 genes were higher in GnRHa treated myoma (e.g. Leukotriene b4 receptor). These observations suggest that a relatively large number of genes are involved in fibroid tumorigenesis and hormone regulation in leiomyoma. These genes could be diagnostic or prognostic markers and might be very useful as new therapeutic candidates.

Conclusions

According to such interesting microarray data, many applications can be imagined, including new diagnostic markers, therapeutic candidates, drug targets for fertilization control, PGD, analysis of stem cell differentiation, and therapy in endometriosis and leiomyoma. However, are these gene profiles all correct? This question has been raised based on the fact that several similar studies performed using almost the same tissues, derived results with significantly different gene expression patterns (Table 2). So which gene list is correct? The answer is that no single list is sufficient. It is reasonable to suspect that subtle variations in patient characteristics or laboratory conditions could dramatically alter microarray results.[45] Although transcript profiling can provide a powerful research tool and attractive data, a careful post-analysis follow-up and validation of microarray experiments will be needed soon. Several available techniques should be involved in confirming these data, including real time quantitative RT-PCR, Northern blotting, Western blotting, ELISA etc. By focusing on confirmed genes, we can hope to identify required pathways for physiological developments or pathogenetic processes and thereby make these gene profiles useful in practice.

For scientists, to obtain gene profiles alone is not satisfactory; studying the functional genomics is more important and meaningful. Several "gain or lost" techniques could be very helpful for following studies, both "in vivo" and "in vitro" after microarray analysis, including a transgenic model, a knock-out model and a knock-down siRNA system. Nevertheless, microarrays are still potentially powerful tools to address the need for high-throughput analysis. Decrease in cost and complexity may facilitate increased availability of this technology in the physician's office and future applications may then

allow tailoring of clinical diagnosis and targeted therapeutic strategies.

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基因微陣列在生殖醫學的應用

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近來微陣列廣泛的應用在生殖醫學上，本文將介紹微陣列在卵子受精、早期胚胎發育、著床及其他不孕症疾病的討論。(長庚醫誌 2006;29:15-24)

關鍵字：微陣列，胚胎，子宮內膜異位，子宮肌瘤。

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