

Microarray technology offers a novel tool for the diagnosis and identification of therapeutic targets for male infertility

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Abstract

Male infertility is now a major reproductive health problem because of an increasing number of environmental pollutants and chemicals, which eventually result in gene mutations. Genetic alterations caused by environmental factors account for a significant percentage of male infertility. Microarray technology is a powerful tool capable of measuring simultaneously the expression of thousands of genes expressed in a single sample. Eventually, advances in genetic technology will allow for the diagnosis of patients with male infertility due to congenital reasons or environmental factors. Since its introduction in 1994, microarray technology has made significant advances in the identification and characterization of novel or known genes possibly correlated with male infertility in mice, as well as in humans. This provides a rational basis for the application of microarray to establishing molecular signatures for the diagnosis and gene therapy targets of male infertility. In this review, the differential gene expression patterns characterized by microarray in germ and somatic cells at different steps of development or in response to stimuli, as well as a number of novel or known genes identified to be associated with male infertility in mice and humans, are addressed. Moreover, issues pertaining to measurement reproducibility are highlighted for the application of microarray data to male infertility.

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Introduction

Microarray technology, including cDNA and oligonucleotide microarrays, has the potential to measure simultaneously thousands of genes expressed in a biological sample, such as cultured cells or dissected tissues. For small samples, the targets can be expanded using RNA amplification (Guatelli *et al.* 1990, Van Gelder *et al.* 1990) or PCR-based cDNA amplification technology (Petalidis *et al.* 2003). This makes it possible to start with as few as 100 cells, a fine biopsy measuring 1 mm×1 mm, or a minimum of 200 ng total RNA, and obtain the labeled targets sufficient for hybridization to an array (Fox *et al.* 2003).

Male infertility is now a major reproductive health problem and the field has attracted considerable attention from scientists and clinicians (Skakkebaek 2003). Worldwide, infertility affects approximately 15% of couples (Nishimune & Tanaka 2006) vs 8% in the US (Leke *et al.* 1993), and more importantly, in half the couples, infertility can be traced to the male (De Kretser & Baker 1999, Nishimune & Tanaka 2006). In some regions, for example in Africa, infertility is around 30–40% and men account for 30% of this

infertility (Leke *et al.* 1993). What is even more disturbing is that the quality of human sperm is decreasing because of environmental pollutants and chemicals, which is believed to cause gene mutations (Duty *et al.* 2003, Sharpe & Irvine 2004). Therefore, genetic disorders might account for a significant percentage of male infertility. It has been reported that 17% of patients with male infertility have abnormal genes (Aoki & Carrell 2003, Rucker *et al.* 1998) and 17% of severe male infertile patients show an abnormal content of protamine 2 in sperm (Carrell & Liu 2001). In addition, severe oligozoospermia and azoospermia are known consequences of some microdeletions (small interstitial deletions) in the azoospermia factor regions of the Y chromosome or in other chromosomes (Vogt *et al.* 1996, Vogt 1997, Escalier 2001). Further, in a number of patients with male infertility, the cause is congenital (Vogt 1995, Vogt *et al.* 1996), but there is still a poor understanding of the genetic defects underlying this abnormality. Thus, the knowledge of the differences in gene expression between normal men and patients with male infertility is essential for understanding male-factor infertility. Microarray technology is a powerful tool for

detecting the changes in gene expression between normal and infertile men. If the accuracy of using differences in gene expression profiles to distinguish patients with male infertility from normal men can be established, microarray technology can be utilized to provide molecular signatures for the clinical diagnosis and gene therapy of male infertility.

In this review, we focus on discussing the differentially expressed genes in male germ and somatic cells at different steps of development, or in response to stimuli, and in transgenic mice compared with normal control mice (Table 1). We also review a number of novel or known genes identified by microarray technology to be correlated with male infertility in mice and humans (Table 2), and then underscore the reproducibility in the usage of microarray technology as a tool for detecting male infertility.

Differentially expressed genes characterized by microarray technology in male germ and somatic cells at different steps of development

Microarray technology has been used to compare gene expression profiles in mitosis, meiosis, and postmeiotic steps during rodent spermatogenesis and a large number of genes have been demonstrated to be differentially expressed during these unique processes. Indeed, using the Affymetrix Genechip system containing approximately 36 000 gene transcripts, Shima and colleagues delineated thousands of unique transcripts that were shown to be differentially transcribed in the mouse testis during the progression of spermatogenesis (Shima *et al.* 2004). This time-course analysis of testis development can be used to reveal the temporal sequence of gene

activation and unlock the underlying regulatory networks and pathways required for normal spermatogenesis and male fertility. Similarly, thousands of genes were also characterized to be differentially expressed at the mitotic, meiotic, and postmeiotic steps of rat spermatogenesis (Schlecht *et al.* 2004). It is noteworthy that more than 290 unknown gene transcripts were identified and some of them were potentially essential for male fertility, since they displayed strong expression throughout meiotic and postmeiotic steps of spermatogenesis. In addition, a large number of gene transcripts were demonstrated to be meiotically and postmeiotically upregulated during or after mouse meiosis, and significantly, 17 out of 19 genes with targeted disruption by homologous recombination were shown to be essential for normal male fertility (Schultz *et al.* 2003). In one study on gene expression during mouse spermatogenesis, cell-specific gene-expression profiles, including the pachytene cluster, the spermatid cluster, and the non-germ cell cluster, were also identified and characterized by microarray analysis and other technologies (Almstrup *et al.* 2004a). In another study on both mice and human, more than 100 genes were shown to be differentially expressed at different stages of testis development, and 42 out of these genes were novel and may encode proteins, important for male fertility (Sha *et al.* 2002). We also discovered hundreds of genes that were differentially transcribed at distinctive steps of male germ cell development, i.e. type A spermatogonia, pachytene spermatocytes, and round spermatids (Pang *et al.* 2003). Several hundred unique unigene clusters specific in type A spermatogonia, pachytene spermatocytes, and round spermatids, were recognized, and more importantly, numerous novel gene transcripts were found to be most abundantly expressed

Table 1 Summary of studies using microarray analysis to characterize the differentially expressed genes in germ and somatic cells at different steps of development or in response to stimuli, and in transgenic mice.

Species	Tissue/cell type/transgenic mice	Microarray employed	References
Human	Adult testes vs fetal testes	cDNA microarray	Sha <i>et al.</i> (2002)
Mouse	Type A spermatogonia vs pachytene spermatocytes vs spermatids	cDNA microarray	Pang <i>et al.</i> (2003)
	Testes from different postnatal day and somatic cells	Oligonucleotide microarray	Schultz <i>et al.</i> (2003)
	Testes from different postnatal day	cDNA microarray	Almstrup <i>et al.</i> (2004b)
	FSH-treated vs untreated testes from hypogonadal mice	Oligonucleotide microarray	Sadate-Ngatchou <i>et al.</i> (2004a)
	TP-treated vs untreated testes from hypogonadal mice	Oligonucleotide microarray	Sadate-Ngatchou <i>et al.</i> (2004b)
	Testes from different postnatal day, Sertoli cells, myoid cells, various germ cells	Oligonucleotide microarray	Shima <i>et al.</i> (2004)
	Testes from MSYq-defective vs normal control	cDNA microarray	Ellis <i>et al.</i> (2005)
	GDNF-treated type A spermatogonia vs untreated type A spermatogonia	Oligonucleotide microarray	Hofmann <i>et al.</i> (2005)
	Testes from MSYq-defective vs normal control	cDNA microarray	Toure <i>et al.</i> (2005)
	TP or dihydrotestosterone-treated testes vs untreated testes	Oligonucleotide microarray	Zhou <i>et al.</i> (2005)
Rat	FSH-treated vs untreated Sertoli cells	Oligonucleotide microarray	McLean <i>et al.</i> (2002)
	SG, SC, ST, SE, and total testes	Oligonucleotide microarray	Schlecht <i>et al.</i> (2004)
	Progenitor, immature, and adult Leydig cells	cDNA microarray	Ge <i>et al.</i> (2005)
	FSH antibody vs control antibody-treated testes	Oligonucleotide microarray	Meacham <i>et al.</i> (2005)

vs, versus; FSH, follicle-stimulating hormone; TP, testosterone propionate; MSYq, the Y-chromosome long arm; GDNF, glial cell line-derived neurotrophic factor; SG, spermatogonia; SC, spermatocytes; ST, spermatids; SE, Sertoli cells.

Table 2 Summary of novel and known genes identified by microarray analysis to be corrected with male infertility.

Species	Tissue and cell types	Novel or known genes	Reference
Human	Adult testes vs embryo testes	tsMACK	Cheng <i>et al.</i> (2002)
	Adult testes vs embryo testes	NYD-SP16	Cheng <i>et al.</i> (2003)
	Adult testes vs fetal testes	NYD-SP12	Xu <i>et al.</i> (2003a)
	Adult testes vs fetal testes vs spermatozoa	PKH-T	Fang <i>et al.</i> (2004)
	Normal testes vs azoospermic testes	Rap1A	Yang <i>et al.</i> (2004)
	Adult testes vs embryo testes vs sperm	BRDT-NY	Zheng <i>et al.</i> (2005a)
	Adult testes vs embryo testes	BGR-like gene	Zheng <i>et al.</i> (2005a)
Mouse	Caput epididymidis of c-ros knockout mice vs wild-type mice	EAAC1	Wagenfeld <i>et al.</i> (2002)
	Epididymidis of c-ros knockout mice vs wild-type mice	NaP _i -IIb	Xu <i>et al.</i> (2003b)
	Normal testes vs infertile testes	Itga6, Itgb1, Maged, Rbm3, and Vim	Ellis <i>et al.</i> (2004)
	Normal testes vs XXSxb testes	Dazl and Taf7l	
	Normal testes vs Bax ^{-/-} testes	Aard, Akr1b3, and Defb119	
	Testes from wild-type vs Dazl knockout mice	Syce1 and Cesc1	Maratou <i>et al.</i> (2004)
	Testes from MSYq-defective vs control mice	Sly	Toure <i>et al.</i> (2005)

vs, versus; MSYq, the Y-chromosome long arm.

in these three types of germ cells (Pang *et al.* 2003). Additionally, a number of the proliferation-related genes were demonstrated by cDNA microarray analysis to be downregulated, while more differentiation-related genes were shown to be upregulated over the course of rat Leydig cell development, including progenitor, immature, and adult Leydig cells (Ge *et al.* 2005). This gene expression profiling can offer novel insights into the molecular mechanisms regulating the development of Leydig cells and other testicular functions, such as steroidogenesis. Together, these enormous resources of gene expression profiles of germ and somatic cells at different developmental steps characterized by microarray technology during spermatogenesis may be helpful to unravel the molecular mechanisms of spermatogenesis and the genetic etiology of male infertility.

Differential gene expression characterized by microarray technology in male germ and somatic cells in response to stimuli

Another application of microarray technology to spermatogenesis and male infertility is to compare gene expression profiles in germ or somatic cells with and without hormonal or growth factor treatment. There are many hundreds of genes showing differential expression patterns in the neonatal mouse testes treated with testosterone propionate (TP) for different time compared with control testes derived from 8-day-old mice (Zhou *et al.* 2005). However, in the testes of hypogonadal mice with TP treatment, relatively fewer genes were differentially expressed at the corresponding treatment time (Sadate-Ngatchou *et al.* 2004b). It is noteworthy that more genes were downregulated than upregulated in mouse testes during the short-time treatment (<24 h) with TP, suggestive of a role for TP in suppressing gene expression. In contrast, more genes were shown to be upregulated by follicle-stimulating hormone (FSH) in testes of

hypogonadal mice, reflecting a primary role of FSH in increasing gene expression (Sadate-Ngatchou *et al.* 2004a). Furthermore, hundreds of genes were shown to be differentially expressed by microarray analysis in rat Sertoli cells after FSH treatment *in vitro* for different time (McLean *et al.* 2002). The gene transcripts regulated by FSH *in vivo* were also identified in rats from 14 to 18 days *post partum* by oligonucleotide microarray, and the FSH-targeted genes may be useful to shed light on the molecular basis of pathological changes in testis, including male infertility (Meachem *et al.* 2005). In glial cell line-derived neurotrophic factor family receptor α -1 (GFR α -1)-positive type A spermatogonia, treatment with glial cell line-derived neurotrophic factor (GDNF), thousands of genes were demonstrated to be differentially expressed as compared with the GDNF untreated control (Hofmann *et al.* 2005), and some of the genes with differential expression were related to cellular proliferation, differentiation/development, or stem cell fate. These experiments regarding the profiles of hormone and growth factor-regulated genes in the whole testis or in isolated germ or somatic cells should allow for a more thorough understanding of the influence of hormones and growth factors upon spermatogenesis, as well as on the pathogenesis of male infertility.

Novel or known genes identified by microarray technology to be correlated with male infertility in mice

The comparison of infertile and normal states, using microarray technology, allows for the rapid discovery of novel or known candidate genes affecting male fertility. In the infertile c-ros receptor tyrosine-kinase knockout mice, the expression of glutamate transporter excitatory amino acid carrier 1 (EAAC1) was shown to be completely absent (Wagenfeld *et al.* 2002), whereas the sodium-inorganic phosphate co-transporter (NaP_i-IIb)

was overexpressed (Xu *et al.* 2003b), suggesting that the abnormal expression of EAAC1 and NaP_i-IIb may play potential roles in male infertility in that the c-ros gene knockout mice are sterile. In the wild-type mouse testes, synaptonemal complex central element 1 (Syce1) and central element synaptonemal complex 1 (Cesc1) were shown to be expressed at increasing levels between days 7 and 11 *post partum*, but it is not the case in the deleted in azoospermia (DAZ)-deficient mice (Maratou *et al.* 2004). SYCE1 and CESC1 protein encoded by Syce1 and Cesc1 respectively have been demonstrated to be recruited by synaptonemal complex protein 1 (SYCP1) and play a putative role in the assembly, stabilization, and recombination of the synaptonemal complex, a key step for meiosis (Costa *et al.* 2005), suggesting that Syce1 and Cesc1 play possible roles in regulating spermatogenesis and mammalian fertility.

Genetically male infertile mice were also used as models to examine the molecular signatures that distinguish pathological and normal states. Four mouse models of male infertility, including XXSxr^b male mice, mshi homozygotes, Bax^{-/-} male mice, and bs homozygotes, were employed to compare their abnormal gene expression profiles with normal fertile mice during the first wave of spermatogenesis using microarray analysis. The results demonstrated that integrin $\alpha 6$ (Itga6), integrin $\beta 1$ (Itgb1), melanoma antigen, family D (Maged), RNA-binding motif protein 3 (Rbm3), and vimentin (Vim) transcripts specific in Sertoli cells, were overexpressed in all four defective models (Ellis *et al.* 2004). On the contrary, a number of spermatogonial gene transcripts, including deleted in azoospermia-like (Dazl) and TAF7-like RNA polymerase II, TATA box-binding protein-associated factor (TAF7l), were underexpressed in XXSxr^b males; while somatic gene transcripts, such as alanine and arginine rich domain containing protein (Aard), aldo-keto reductase family 1, member B3 (Akr1b3), and defensin, β 119 (Defb119), were overexpressed in Bax^{-/-} male mice. This suggests that overexpression of these somatic gene transcripts or underexpression of spermatogonial gene transcripts play potential roles in the progress of mouse male infertility. Furthermore, dozens of the Y-chromosome long arm (MSYq)-encoded transcripts were also shown to be significantly reduced in the three MSYq-deficiency mouse models, including 2/3 MSYq⁻, 9/10 MSYq⁻, and MSYq⁻ mice (Toure *et al.* 2005). Among them, Sycp3-like Y-linked (Sly), encoding a putative protein homologue to the proteins XLR and XMR that are coded by X-chromosome relatives, should be highlighted since Sly deficiency is the more likely cause of the abnormality in sperm head shape found in males with MSYq-deficiencies. These data suggest that microarray technology is an effective tool to identify novel candidate genes that contribute to male infertility. Based on a different normalization method of microarray data, Ellis and colleagues demonstrated that the majority (more than

65%) of the upregulated transcripts in the same three MSYq-deficiency models was from the X- or Y-linked copies (Ellis *et al.* 2005). The above-mentioned studies illustrate that microarray technology can be used to identify X- or Y-chromosome-encoded transcripts, and that abnormal expression (downregulation or upregulation) of these genes is likely to cause male mouse infertility. Due to a close similarity between mouse and human spermatogenesis, microarray analysis of different gene expression profiles between the mouse models of male infertility compared with normal fertile mice can shed light on the identification of novel genes important in human male infertility.

Novel genes identified by microarray technology to be correlated with male infertility in humans

Microarray technology is also being used to identify new genes that may be closely correlated with male infertility in humans. Through constructing a human adult testis cDNA microarray, Cheng and colleagues identified a novel testis-specific gene tsMCAK, homologue to gene HsMCAK, encoding human testis-specific mitotic centromere-associated kinesin (Cheng *et al.* 2002). tsMCAK was expressed in the testes of patients with spermatogenic arrest at the spermatocyte step, but it was absent in the testes of infertility patients with Sertoli-cell-only syndrome or in the testes of infertility patients with spermatogenic arrest occurring in spermatids, indicating that tsMCAK may be involved in the later steps of spermatogenesis and that lost expression of tsMCAK may be correlated with male infertility. Subsequently, they identified another novel human testicular gene called NYD-SP16, which encodes probably the spermatogenesis-associated nine protein (Cheng *et al.* 2003). It is notable that NYD-SP16 was absent in the testes of infertile patients with Sertoli-cell-only syndrome (Cheng *et al.* 2003). Likewise, Xu and colleagues characterized a new spermatogenesis-related gene, NYD-SP12, which encodes a testis-specific Golgi protein (Xu *et al.* 2003a). NYD-SP12 was primarily expressed in spermatocytes and spermatogonia, but not in somatic cells in mice at different ages, and more importantly, NYD-SP12 transcript was not detected in all the patients with spermatogenic arrest or Sertoli-cell-only syndrome. Further, a novel pyridoxal kinase mRNA splice variant, PKH-T, was shown to be highly expressed in adult human testis and spermatozoa, but was absent in the testis of patients with Sertoli-cell-only syndrome and in some patients with spermatogenic arrest (Fang *et al.* 2004). In short, the abnormal expression of these genes mentioned above in infertile men or patients with spermatogenic arrest may be correlated with male infertility. In 2005, two new genes, including bubblegum-related-like (BGR-like) and BRDT-NY genes, an alternative splice

variant of BRDT gene (bromodomain, testis-specific), were also identified and characterized through microarray analysis (Zheng *et al.* 2005a, 2005b). Gene BGR encodes bubblegum-related protein and gene BRDT codes testis-specific bromodomain protein. Intriguingly, neither BRDT-NY nor BGR-like gene transcript was detected in the testis of certain azoospermic patients, suggestive of potential roles of both genes in the development of azoospermia. In addition, hundreds of genes were differentially expressed in the testes between fertile and azoospermic patients (Yang *et al.* 2004); among these genes, the mRNA expression of RAS-related protein-1A (Rap1A), a member of the RAS oncogene family, was higher in the azoospermic testes compared with the normal testes, suggesting that Rap1A might play a role in the process of azoospermia. Taken together, these studies demonstrate that microarray technology is feasible for the identification of male infertility-related candidate genes that are likely to provide molecular signatures for the diagnosis and gene therapy for male infertility in the future. Aberrant expression of these novel genes may result in male infertility, and thus exploration of these specific genes could also account for the pathological mechanisms of male infertility (Okabe *et al.* 1998). An important caveat is that the data obtained from human samples so far show no direct comparison between normal men and patients, since they do not have well-characterized genetic differences, while it is the case, for example, with mouse Y-chromosome depletion models (Ellis *et al.* 2005, Toure *et al.* 2005).

On the other hand, microarray analysis was used to address other aspects of human testicular dysgenesis syndrome, such as testicular carcinoma *in situ* (CIS). One of the largest microarrays was employed to identify several hundred gene transcripts that were upregulated in testicular tissue from CIS as compared with normal testicular parenchyma (Almstrup *et al.* 2004b). These microarray data are likely to offer novel candidates for diagnostic markers for CIS and account for the pluripotency of CIS, since a range of embryonic stem cell markers, including NANOG, a homeobox transcription factor, and POU5F1, an octamer-binding transcription factor, is highly expressed in CIS. Meanwhile, microarray analysis was also employed to characterize expression changes of numerous novel genes and oncogenes in testicular germ cell tumors (TGCT) and testicular seminomas (Skotheim *et al.* 2002, Yamada *et al.* 2004), and this should be helpful to identify new treatment targets for TGCT and seminoma and also shed light on the tumorigenesis of these conditions.

Reproducibility is essential for the application of microarray data to male infertility

Microarray analysis comprises a multi-step process. The basic procedure of oligonucleotide microarray, for

example, involves the extraction and purification of total RNA or mRNA from normal and abnormal samples, the synthesis and cleanup of double-strand cDNA and biotin-labeled cRNA that is subsequently fragmented for the targets. The fragmented cRNA is then hybridized to a Genechip containing tens and thousands of cDNAs or oligonucleotides, after which the excess is washed off and the microarray can be scanned under a laser light for the signal of individual probes. The raw data are acquired and analyzed using appropriate software. Each step contributes to the quality of the data in the microarray analysis. Certain significant issues associated with microarray analysis, including experiment design, target preparation, array hybridization, data analysis and validation, have been described in detail elsewhere (White & Salamonsen 2005).

It is worth considering four major characteristics, namely the absolute expression levels, the subtractive degree of change, the fold changes, and the reproducibility of the data when comparing the gene expression profile between two samples using microarray technology (Butte 2002). Among these aspects, reproducibility is of paramount importance. One way to show the reproducibility of microarray analysis is to repeat the experiments at least two or three times. Wodicka and colleagues have reported that 99.8% of genes have a similar expression pattern when a sample is hybridized to two identical arrays, and 99% similarity can be obtained when independent samples are employed (Wodicka *et al.* 1997). In order to confirm the reproducibility of microarray technology, we compared the gene expression profiles of the seminiferous tubules derived from adult mice using oligonucleotide microarray. We made two independent preparations of RNA targets and used 15 µg biotin-labeled cRNA for hybridization with the mouse Genechips containing 12 488 gene transcripts. Microarray analysis revealed that 6729 gene transcripts were present in both seminiferous tubule preparations, and only 17 out of 6729 transcripts were differentially expressed between these two samples (Fig. 1), indicating that 99.72% of transcripts have a very similar expression profile. Significantly, a high reproducibility for the mouse expression profile of differentially regulated genes was also acquired in two different laboratories (Schultz *et al.* 2003, Shima *et al.* 2004), and the oligonucleotide microarray data are highly reproducible in similar genes between different species, such as mice and rats (Schultz *et al.* 2003, Shima *et al.* 2004, Wrobel & Primig 2005).

Another way to test the reproducibility of microarray data is to confirm the mRNA expression of differentially expressed genes of particular interest by existing molecular technologies, such as real-time reverse transcriptase (RT) and PCR (real-time RT-PCR), semi-quantitative RT-PCR, differential display competitive PCR, northern blots, and RNA *in situ* hybridization. Ellis *et al.* identified hundreds of transcripts that were differentially

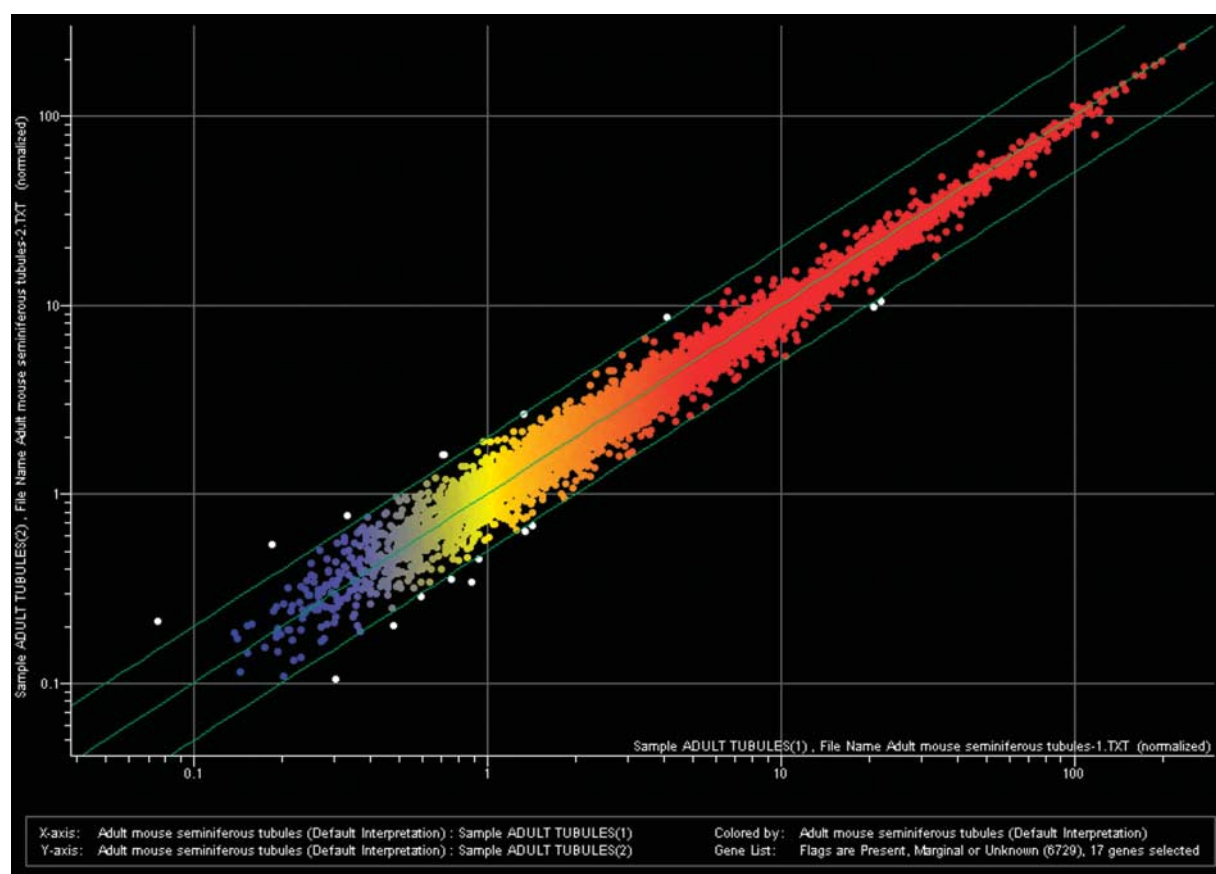


Figure 1 Scatter plot comparison of gene expression pattern in two samples of adult mouse seminiferous tubules. Each dot represents a specific gene expression present in adult mouse seminiferous tubules. Gene transcripts that were upregulated or downregulated more than two-fold were considered to be differentially expressed. Gene transcripts with no change were represented along the center green line. Gene transcripts that were upregulated more than two-fold were shown above the upper green line, and gene transcripts that were downregulated more than two-fold were shown below the lower green line. There were only 17 transcripts that were shown to be differentially expressed between two independent samples as highlighted by the white dots.

expressed during the first wave of mouse spermatogenesis, and they obtained the confidence of data quality by real-time RT-PCR (Ellis *et al.* 2004). They also verified the upregulated gene transcripts in MSYq-deficient mice by northern blotting and underpinned the reproducibility of the microarray data in sex chromosome-linked genes using real-time RT-PCR (Ellis *et al.* 2005). Almstrup and colleagues combined a number of methodologies, including microarray, differential display competitive PCR, and *in situ* hybridization, to identify three groups of cell-type-specific gene-expression profiles during spermatogenesis (Almstrup *et al.* 2004a). RNA *in situ* hybridization was also employed to confirm the microarray data showing that Sly transcript was predominantly expressed in spermatids (Toure *et al.* 2005). In our laboratory, we corroborated the microarray data showing col1a1 and col1a2 mRNA expression levels in the seminiferous tubules of immature and adult mice using semi-quantitative RT-PCR (He *et al.* 2005). Adachi and colleagues revealed that 11 genes were upregulated and three genes were downregulated by diethylstilbestrol in

28-day-old mice using cDNA microarray analysis, however, they only confirmed eight out of 11 upregulated genes by real-time RT-PCR (Adachi *et al.* 2002). Considered together, these examples illustrate that microarray technology is reliable and robust (Wrobel & Primig 2005), but great attention must be paid to reproducibility when microarray technology is used as a tool to identify and characterize novel genes that are possibly correlated with male infertility. Furthermore, since mRNA expression profiles using microarray analysis of two samples cannot reveal translational controls and are unable to completely correlate with protein expression, microarray technology must be integrated with other molecular technologies, including Western blots, flow cytometric analysis, ELISA, and protein arrays.

When the sample size is very small, the RNA must be amplified using linear or exponential amplification methods, including T7-based linear RNA amplification, switching mechanism at the 5'-end of RNA template (SMART) PCR amplification, and global PCR

amplification, to obtain sufficient targets for microarray analysis. On one hand, T7-based linear RNA amplification generated high fidelity microarray data compared with non-amplified mRNA samples (Zhao *et al.* 2002) and SMART PCR and global PCR amplification also produced reproducible results (Livesey 2003, Subkhankulova & Livesey 2006). For example, 94% overlap of the differentially expressed genes between the amplified samples and the unamplified ones was obtainable using linear amplification (Polacek *et al.* 2003). On the other hand, all three amplification methods induced errors in the microarray data (Subkhankulova & Livesey 2006). SMART PCR amplification led to a systematic reduction in the gene-expression ratios compared with the data from the unamplified samples (Livesey 2003, Petalidis *et al.* 2003, Subkhankulova & Livesey 2006), while T7-based linear RNA amplification enhanced the number of differentially expressed genes when compared to the number identified from the unamplified RNA samples (Patel *et al.* 2005). The error of microarray data following amplification depends on many factors, including the amplification protocol (Puskas *et al.* 2002, Jenson *et al.* 2003, Petalidis *et al.* 2003, Wilson *et al.* 2004), the degree of amplification required (Zhao *et al.* 2002), the quality of the starting total RNA template, the concentration of the promoter primer used to synthesize cDNA (Jenson *et al.* 2003), and the time-dependent RNA degradation during T7-based linear RNA amplification (Spiess *et al.* 2003). It is noteworthy that the different amplification protocols resulted in distinct correlations between the amplified targets compared with the unamplified ones (Puskas *et al.* 2002, Petalidis *et al.* 2003). Thus, considerable attention should be paid to the systematic error of microarray data following linear or exponential amplification, and validation studies using the existing molecular methods mentioned above will reduce amplification bias.

Conclusions

Microarray technology allows for rapid access to a more precise diagnosis and better therapeutic strategies of various diseases. The Food and Drug Administration of the United States of America has a growing interest in the use of microarray technology as a diagnostic device. This offers a good opportunity for both scientists and clinicians to improve diagnostics and develop novel approaches of treatment for various male infertility diseases. Thus far, a growing number of novel or known genes have been identified and characterized by microarray technology to be possibly correlated with male infertility in mice and humans, which could reveal novel candidates for both diagnosis and therapy. However, reproducibility of microarray data is essential. If these novel genes in the molecular signature can tell us something about the pathogenesis of male infertility, microarray technology may be used as a clinical diagnostic tool and eventually lead to gene therapy.

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