1 Introduction

1.1 Spermatogenesis and fertilization

1.1.1 *The testis*

The testis is the primary organ involved in male germ cell production. It is under the control of the hypothalamus, which regulates the secretion of the pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). The testis is composed of numerous thin, tightly coiled ducts known as the seminiferous tubules, which comprise about 90 percent of the testicular mass. The main cell types in the testis are Leydig cells, Sertoli cells, and germ cells. The Leydig cells are located outside the walls of the seminiferous tubules and, in response to LH stimulation, produce steroid hormones. These steroid hormones in conjunction with FSH regulate the Sertoli cells, which form a blood-testis barrier through formation of tight junctions. The Sertoli cells, which are often referred to as "nurse cells", are regularly distributed within the walls of the seminiferous tubules and provide an environment for germ cells to develop and mature through the production of survival factors. One factor that mediates the survival and differentiation of germ cells is Stem Cell Factor (SCF), also called Kit-ligand (Packer et al., 1995). The Sertoli cells also help transport young germ cells from the periphery of the seminiferous tubule to the lumen (Griswold, 1995).

1.1.2 *Spermatogenesis*

Spermatogenesis is the sequence of developmental events by which spermatogonial stem cells give rise to functional spermatozoa. This process takes place in the seminiferous tubules of the male testis. In the mammalian testis, spermatogonia arise from primordial germ cells, which migrate into the developing testis during fetal life. Here, they become associated with the differentiating Sertoli cells, and seminiferous cords are formed. In this setting, they transform into gonocytes, which remain centrally placed, surrounded by the immature Sertoli cells. Following a period of multiplication, the gonocytes migrate to the basement membrane of the seminiferous tubule where they divide to form type A spermatogonia (de Kretser et al., 1998; de Rooij, 1998).

Spermatogenesis can be divided into three stages (Figs. 1 and 2):
- Spermatogonial stage – mitotic clonal expansion
- Meiotic stage – production of the haploid gamete
- Spermiogenesis stage – morphological changes of spermatids into spermatozoa
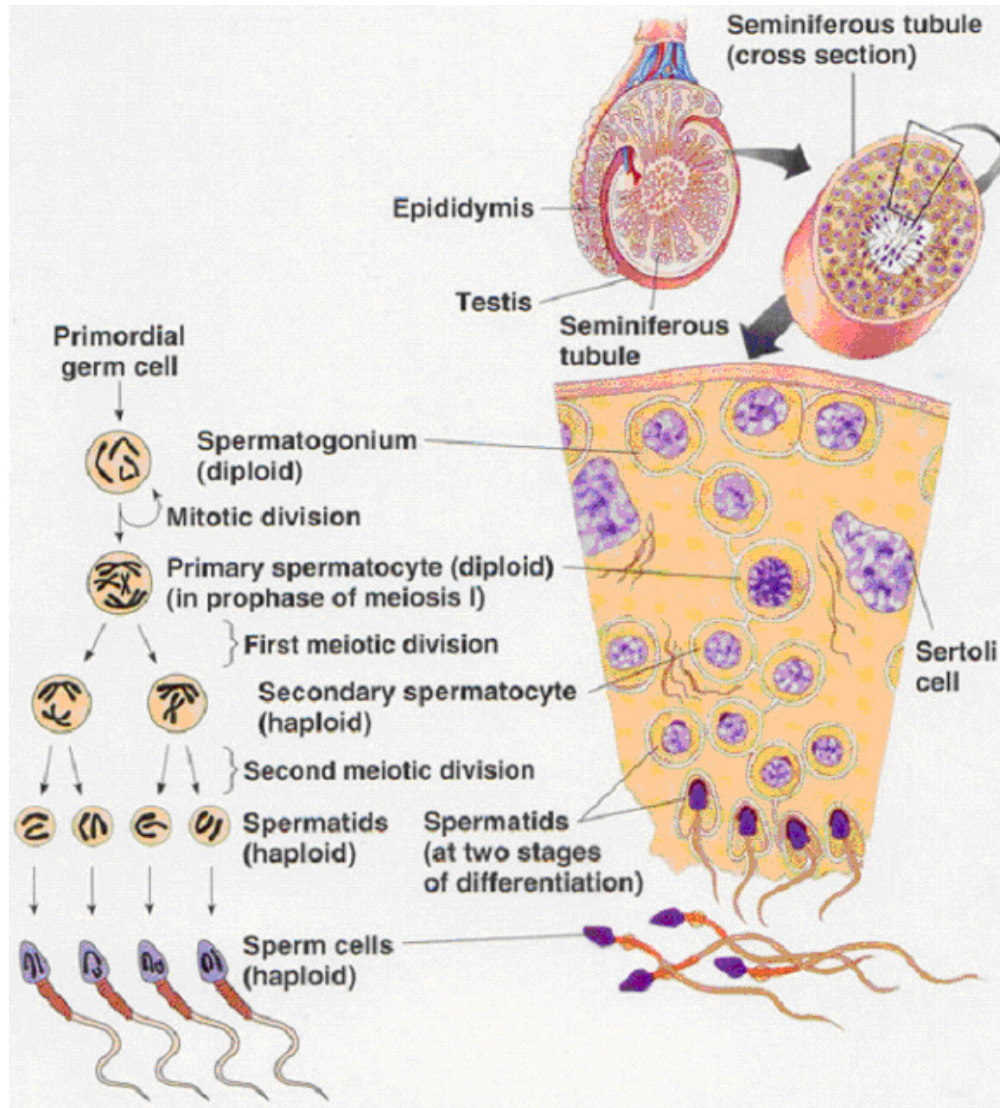
Figure 1. Mammalian spermatogenesis
Spermatogenesis takes place in the seminiferous tubules of the testis. During differentiation, the germ cells, surrounded by Sertoli cells, migrate towards the lumen of the tubules, from where they are released to the epididymis. Spermatogonial stem cells at the basement membrane mitotically divide and give rise to differentiating spermatogonia. From Campbell et al., 1999.

Spermatogonial stage

The first phase of spermatogenesis begins with the division of the spermatogonia that line the seminiferous tubule near the basement membrane. Type A spermatogonia divide to maintain the population of the stem cell pool. Some spermatogonia resulting from these mitotic divisions stay in the resting pool and do not differentiate, while the remaining type A spermatogonia proliferate and undergo several stages of division and differentiation (Oakberg, 1971). The number of spermatogonial cell types identified varies be-

tween species. In the human, type A pale, type A dark and type B spermatogonial cells can be distinguished. In the rodent testis, multiple type A spermatogonia, intermediate and type B spermatogonia have been identified (de Kretser et al., 1998).
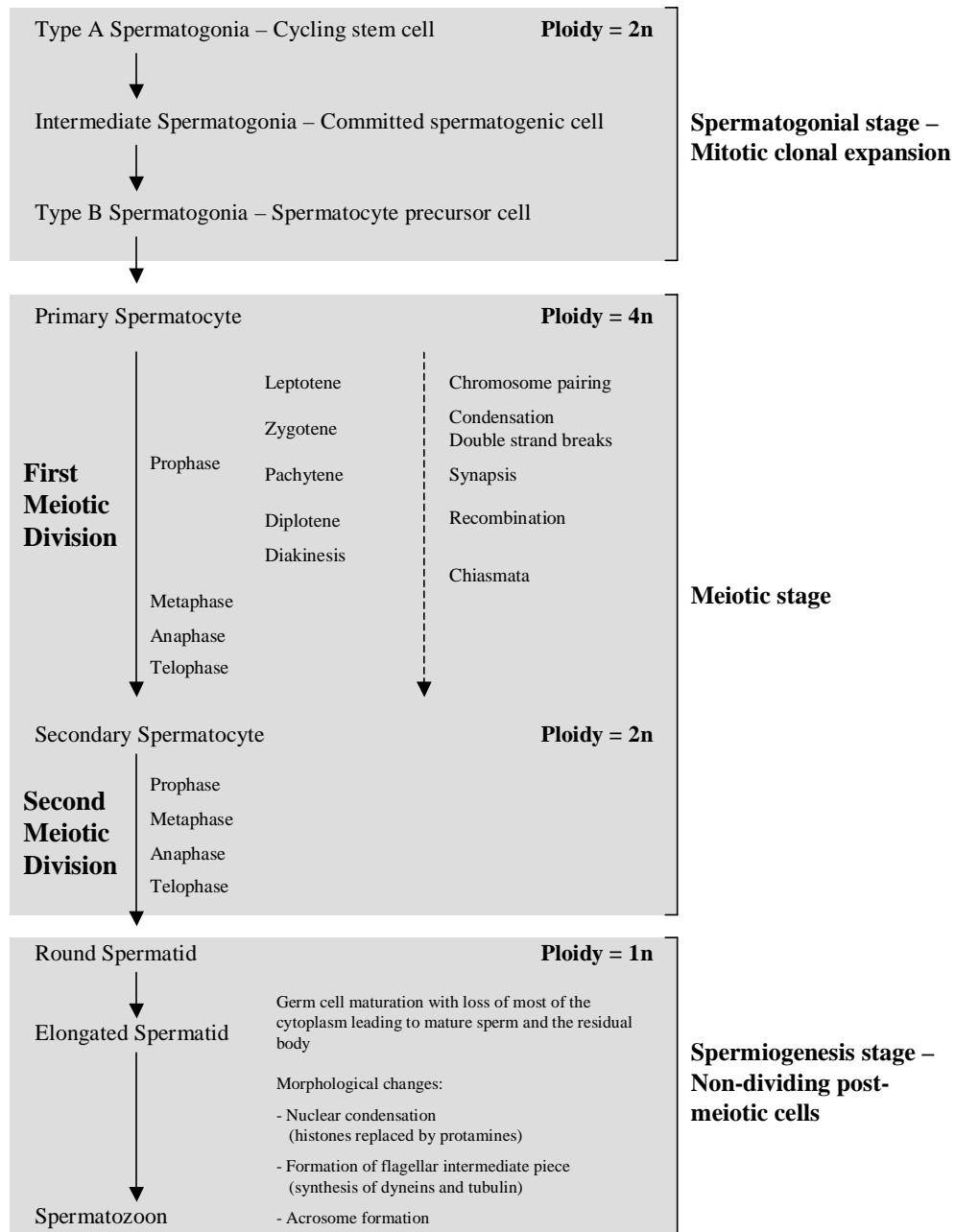
Type A Spermatogonia – Cycling stem cell **Ploidy = 2n**	
↓	
Intermediate Spermatogonia – Committed spermatogenic cell	**Spermatogonial stage –**
↓	**Mitotic clonal expansion**
Type B Spermatogonia – Spermatocyte precursor cell	

↓

Primary Spermatocyte		**Ploidy = 4n**	
	Leptotene	Chromosome pairing	
	Zygotene	Condensation Double strand breaks	
First Meiotic Division	Prophase Pachytene	Synapsis	
	Diplotene	Recombination	
	Diakinesis	Chiasmata	**Meiotic stage**
	Metaphase Anaphase Telophase		
Secondary Spermatocyte		**Ploidy = 2n**	
Second Meiotic Division	Prophase Metaphase Anaphase Telophase		

Round Spermatid	**Ploidy = 1n**
↓	Germ cell maturation with loss of most of the cytoplasm leading to mature sperm and the residual body
Elongated Spermatid	
	Morphological changes:
	- Nuclear condensation (histones replaced by protamines)
	- Formation of flagellar intermediate piece (synthesis of dyneins and tubulin)
Spermatozoon	- Acrosome formation

Spermiogenesis stage – Non-dividing post-meiotic cells

Figure 2. The individual phases of spermatogenesis and their respective cell types
Spermatogenesis can be divided into three distinct phases. Shown are the specific processes and the terms for their respective cell types occurring during these stages.

Meiotic stage

The type B spermatogonia enter the preleptotene stage of the meiotic process. They are then termed primary spermatocytes. The primary spermatocytes (ploidy = 4n) undergo the two divisions of meiosis. During the first meiotic division, each morphologic chromosome (actually two sister chromatids) aligns at the cell equator paired with its homologous partner. During this alignment, recombination between the homologous chromosomes can occur. One homologue (with both sister chromatids) is randomly selected to travel to one daughter cell, the other homologue of that chromosome goes into the other cell. No division of centromeres occurs, however, and the sister chromatids remain together. Cells passing through the prophase of the first meiotic division can be subdivided on a cytological basis into preleptotene, leptotene, zygotene, pachytene, and diplotene stages (see Fig. 2). During the second division the centromeres do divide, as in mitosis, and one sister chromatid (now an independent chromosome) of each morphologic type is apportioned into each haploid daughter cell (ploidy = 1n). The resulting cell is termed round spermatid (de Kretser and Kerr, 1994).

Spermiogenesis stage

In this stage, the round spermatid enters spermiogenesis, the third part of spermatogenesis. During this process numerous nuclear and cytoplasmic changes occur in the spermatids, eventually leading to the formation of the spermatozoa.

Restructuring of the cell includes:

- Nuclear condensation and movement of the nucleus to the periphery of the cell.
- Formation of a modified lysosome known as the acrosome, which becomes attached to the surface of the nucleus in apposition to the cell membrane.
- Flagellar formation, which includes the development of a core of microtubules, the axoneme, which arises from one of the centrioles of the round spermatid. Later modifications of the axoneme lead to the formation of the tail.
- Following the completion of these events, the spermatid sheds a large part of its cytoplasm as the residual body, which is phagocytosed by the Sertoli cell.

The process of spermiogenesis ends with release of the spermatozoa from the Sertoli cell. The spermatozoa are still immotile when released and must mature further during storage and transition in the epididymis (de Kretser et al., 1998).

Spermiogenesis in the rat can be subdivided into 19 well-defined stages (Leblond and Clermont, 1952). In any area of the seminiferous tubule of a rat, there may be one or two generations of spermatids. Spermatids at a given stage are always associated with spermatocytes and spermatogonia at given stages of their respective developments.

Synchronization of the spermatogonial stages

Since a cycle of the seminiferous epithelium consists of the series of changes occurring between two successive appearances of the same cell association in a given area of the tubule, any cycle starting with the formation of spermatids ends when the next genera- tion of spermatids appears in the same area. Leblond and Clermont (1952) therefore de- cided to subdivide the cycle into 14 corresponding periods, and they explicitly defined the cell association of each (Table 1). To avoid confusion, the stages of spermatogene- sis are designated by Arabic numerals, while the corresponding periods of the cycle of the seminiferous tubule are designated by Roman numerals.

Table 1. The 14 stages of the cycle of the seminiferous epithelium in the rat

Stage of cycle		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Spermatogonia Type A, Type B										A	A	A	A	A	A
		A	A	A	B/A	B/A	B/A	A	A						
Spermatocytes I	1st layer							R	R	R/L	L	L	L	Z	Z/T
		T	T	T	T	T									
	2nd layer						P	P	P	P	P	P	P	DI	M
Spermatocytes II															SSM
1st layer of **spermatids**	Phase	Golgi phase			Cap phase				Acrosome phase						
	Stage	1	2	3	4	5	6	7	8	9	10	11	12	13	14
2nd layer of **spermatids**	Phase	Maturation phase													
	Stage	15	16	16	17	17	18	19	19 Release						

Spermatocytes I: R = Resting; L = Leptotene; Z = Zygotene; T = Transition form; P = Pachytene; DI = Diplotene and Diakinesis; M = Metaphase. From Leblond and Clermont, 1952.

1.1.3   Fertilization

Mammalian sperm cells must reside in the female reproductive tract and undergo a series of physiological changes before they are able to penetrate and fertilize the egg. This poorly understood maturation process is called capacitation (Fraser, 1998).

To penetrate the substantial cumulus cell barrier surrounding ovulated eggs of most mammalian species (Fig. 3A), sperm use hyperactivated motility (Yangagimachi, 1994) and a glycosylphosphatidylinositol (GPI)-anchored surface hyaluronidase, named PH-20 (Lin et al., 1994). Hyperactivation is probably driven by CatSper-mediated Ca^{2+} entry (Carlson et al., 2003; Quill et al., 2003). The mammalian egg is further surrounded by an extracellular envelope called the zona pellucida, which sperm must penetrate before they can make contact with the surface of the egg itself (Fig. 3B). The zona pellucida of the mouse egg is a matrix composed of three glycoproteins called ZP1, ZP2 and ZP3. ZP3 functions as a sperm receptor, and its sperm-binding activity is mediated by oligosaccharide side chains (Florman and Wassarman, 1985; Spargo and Hope, 2003). The zona pellucida also serves as a barrier to sperm from heterologous mammalian species.
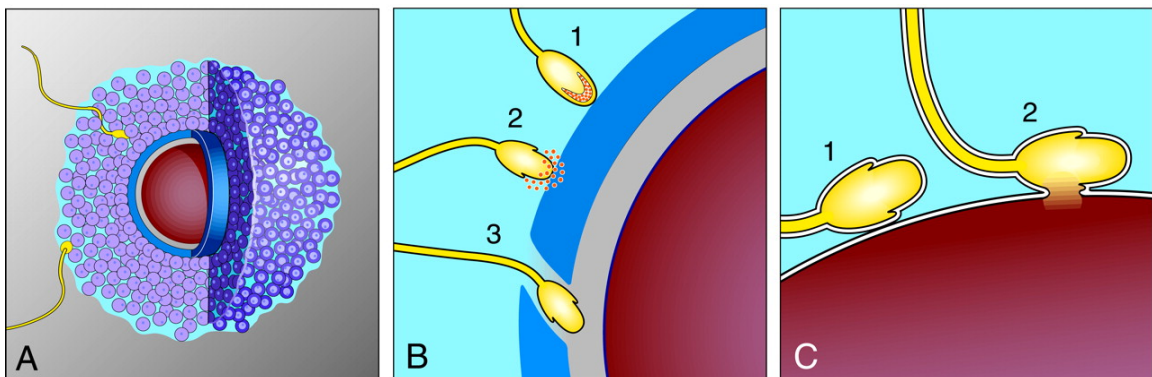

Figure 3. Mammalian fertilization
(A) Sperm penetration of cumulus cells (purple) to reach the zona pellucida (navy blue). (B) Egg depicted with cumulus cells removed; sperm 1 binds to the zona pellucida (navy blue); sperm 2 undergoes exocytosis, releasing acrosomal contents; sperm 3 penetrates the zona pellucida and begins entry into perivitelline space (gray). (C) Sperm 1 binds to the egg plasma membrane by the side of its head, in a central region (equatorial region); sperm 2 fuses with the egg plasma membrane. From Primakoff and Myles, 2002.

Identifying the zona receptors on the sperm surface has proved to be more difficult than identifying the zona ligands, probably because of the great complexity of the sperm surface. A wide variety of sperm surface components are implicated in binding the zona

pellucida. Among the sperm proteins thought to bind ZP3 oligosaccharides in particular, sperm surface β-1,4-galactosyltransferase-I (GalT I) satisfies virtually all criteria expected of a ZP3 receptor (Miller et al., 1992; Talbot et al., 2003). However, recent findings suggest that gamete recognition is more complex than a single receptor-ligand interaction and that a ZP3- and GalT I-independent interaction is involved in gamete adhesion (Rodeheffer and Shur, 2004).

Binding of the sperm to the zona pellucida triggers the acrosome reaction, during which the sperm release the contents of their large secretory granule, the acrosome (see Fig. 3B). The sperm penetrate the zona pellucida through a combination of force and digestion by the enzymes released by the acrosome reaction (Allen and Green, 1997).

Having reached the perivitelline space between the egg ZP and plasma membrane, sperm must bind to the cell membrane and then fuse with it (Fig. 3C). Fusion with a single sperm prevents the egg plasma membrane from fusing with further sperm that have penetrated the zona pellucida. At this point, the egg has been fertilized and becomes a zygote, and other sperm are no longer able to bind to the zona pellucida.

With fertilization, a mammalian egg becomes activated by a series of intracellular Ca^{2+} oscillations that are essential for embryo development. The mechanism by which the sperm induces this phenomenon has long been unknown. Only recently a sperm-specific phospholipase C, PLC ζ, was identified. This phospholipase triggers Ca^{2+} oscillations indistinguishable from those at fertilization (Saunders et al., 2002) and may represent the long elusive "sperm factor" responsible for egg activation.

The fertilization of an egg produces a new individual and, theoretically, brings about an endless series of generations. As a totipotent stem cell, the fertilized egg gives rise to all cell types of the body.

1.2   Gene expression in the testis

Meiosis, haploid gene expression, formation of the acrosome and the flagellum, replacement of histones with protamines, and nuclear condensation are unique processes of spermatogenesis. Spermatogenesis also includes the typical processes of differentiation, including a self-renewing stem-cell population, cell division associated with step-wise developmental processes, and development of highly differentiated cells. The highly ordered process of spermatogenesis requires a precise and well-coordinated program that regulates the constantly changing patterns of gene expression. Some of the genes expressed to serve these different purposes are exclusive to spermatogenic cells but are closely related to genes expressed in somatic cells. Examples are the genes for histone H1t, heat shock protein HSP70-2 and lactate dehydrogenase C. Other genes are unique to spermatogenic cells and have no isoforms in other tissues, e.g. synaptonemal complex protein 1, transition proteins 1 and 2 and protamines 1 and 2 (Eddy et al., 1993; Eddy and O'Brien, 1998).

With the replacement of histones by transition proteins and subsequently by protamines, the transcriptional activity of spermatids is reduced (Sassone-Corsi, 2002). The tightly packed DNA-protamine complexes cease transcription several days before the completion of spermiogenesis, after which translational repression becomes an important feature in the elongating spermatids. Mature spermatozoa are transcriptionally and translationally inactive. However, it has been shown that mature spermatozoa carry a surprising number of different mRNA. Spermatozoal gene expression profiles could therefore be used to distinguish fertile from infertile men. Furthermore, it is postulated that sperm contribute mRNA to the egg during fertilization and that these transcripts are important for the developing embryo (Ostermeier et al., 2002).

Thus far, only a small percentage of the genes expressed in germ cells have been identified and characterized, and a greater number of genes and gene functions remain unknown. Consequently, we do not understand how a male germ cell develops into a mature spermatozoon that is then capable of fertilizing an egg. Components of signal transduction pathways, e.g. the transcription factor CREM (cyclic AMP responsive element modulator (Foulkes et al., 1992)), have been identified in germ cells, but how the different pathways are activated and interact in these cells has yet to be determined.

Furthermore, the underlying basis of fertilization in mammals is still poorly understood. Contributing factors include: a) paucity of eggs, b) heterogeneity of sperm cell populations, c) a lack of information on the number and nature of molecules that serve as potential players (Quill et al., 2001). Unlike many invertebrates and some lower verte-

brates, where sperm cell behavior is highly synchronous, at any given moment only a fraction of mammalian sperm cells appears to respond to components of the egg extracellular matrix, to chemoattractants or to other signaling molecules (Lee et al., 1987; Eisenbach, 1999). Thus, sensitive functional bioassays have been difficult or impossible to establish. One attempt to overcome this problem could be the identification of all proteins potentially expressed in the spermatozoon. To make a list of potential molecules involved in fertilization more experimentally palatable, an initial list of potential players could be restricted to proteins expressed exclusively by male germ cells. Additionally, since proteins involved specifically in fertilization are likely to be expressed late in spermatogenesis, analysis can be focused on gene products expressed during meiosis or later. To this end, the Garbers laboratory initially generated signal peptide trap cDNA libraries from spermatid-enriched cell populations and successfully identified a number of important sperm proteins such as CatSper2 (Quill et al., 2001). Based on these screens it was estimated that more than 200 unique sperm proteins existed; even this was somewhat surprising given 'leaky transcription' has long been attributed to the testis, suggesting that a large number of sperm transcripts may be shared with other cells (Garbers, 2000).

Recent high throughput efforts by other groups resulted in the identification of a few new testis-specific transcripts. For example, 19 novel pre-meiotic male germ cell-specific genes were identified by cDNA subtraction (Wang et al., 2001), and use of a differential display reverse transcriptase-polymerase chain reaction resulted in the identification of other genes expressed during spermatogenesis (Anway et al., 2003). Using cDNA microarrays in mouse or human, four (Tanaka et al., 2002), and 42 (Sha et al., 2002) novel genes potentially involved in the regulation of spermatogenesis have been identified. The most comprehensive gene expression study to date used testis-specific cDNA microarrays to study gene expression in the testis of wild-type and *Dazl* knockout mice (Maratou et al., 2004). The study describes the expression patterns of approximately 1000 genes that are expressed during mouse spermatogenesis. Even though these approaches identified a number of new genes potentially important in spermatogenesis, the studies failed to determine the potential number or identity of genes specifically expressed in the male germline that serve as likely participants in germ cell development and fertilization.

1.3   The spermatogonial stem cell

Spermatogonial stem cells in the testis originate from primordial germ cells (PGCs). During development, these cells arise from embryonal ectoderm, from where they migrate to the genital ridges. In females, the PGCs undergo meiosis and become oocytes, thereby ending their stem cell potential. In males however, these cells retain their stem cell potential. They enter fetal seminiferous tubules and become gonocytes (de Rooij, 1998). Following birth, the gonocytes migrate to the basement membrane of the seminiferous tubules and differentiate into spermatogonial stem cells. Spermatogonial stem cells share two characteristics with other adult stem cells: they can self-renew and provide daughter cells, which can differentiate into terminal cell types (van der Kooy and Weiss, 2000). Spermatogonial stem cells constitute only a small fraction of the testicular germ cells, approximately 30,000 per mouse testis. The stem cells are located in the most peripheral region of the seminiferous tubule (de Rooij and Grootegoed, 1998). In rats and mice, type A-single (A_s) spermatogonia, formerly classified as "A-stem" spermatogonia (Oakberg, 1971) or "A-isolated" spermatogonia (Huckins, 1971b; Huckins, 1971c) are considered to contain the spermatogonial stem cell population (Brinster, 2002; Zhao and Garbers, 2002). They continuously undergo asymmetric mitotic divisions and generate the first differentiated cell type, the A_1 spermatogonia.

The current technique to evaluate spermatogonial stem cell character is the transfer of cells to recipient males and determination of testis colonization activity (Fig. 4), a method that can take weeks or even months for evaluation (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Dobrinski et al., 1999; Nagano et al., 1999). Thus, the necessity of using colonization of a recipient testis as an assay for spermatogonial stem cell activity significantly slows down spermatogonial stem cell studies. The spermatogonial stem cell in mammals has not been unequivocally identified through the use of germ cell-specific molecular markers (Huckins, 1971b; Huckins, 1971c; Oakberg, 1971; de Rooij, 2001), making testicular transplantations necessary. Although testicular cells characterized by certain surface markers (integrin-$\alpha 6^+$, integrin-$\beta 1^+$, Thy-1^+, CD24$^+$, CD9$^+$, Kit$^-$, integrin-αv^-, MHC-Ia/β2M$^-$, Sca-1^- and CD34$^-$) have been sorted and are enriched in stem cell activity (Shinohara et al., 1999; Shinohara et al., 2000b; Kubota et al., 2003; Kanatsu-Shinohara et al., 2004), these gene products are also present in other stem cells, differentiated germ cells, and testicular somatic cells. Various other molecular markers for germ cells also exist (e.g. *Dazl, Vasa*), but these genes are expressed at many stages of development (Reijo et al., 2000; Noce et al., 2001) and therefore are not unique markers of the spermatogonial stem cell. Thus, at present no specific markers are available for the selection of spermatogonial stem cells.
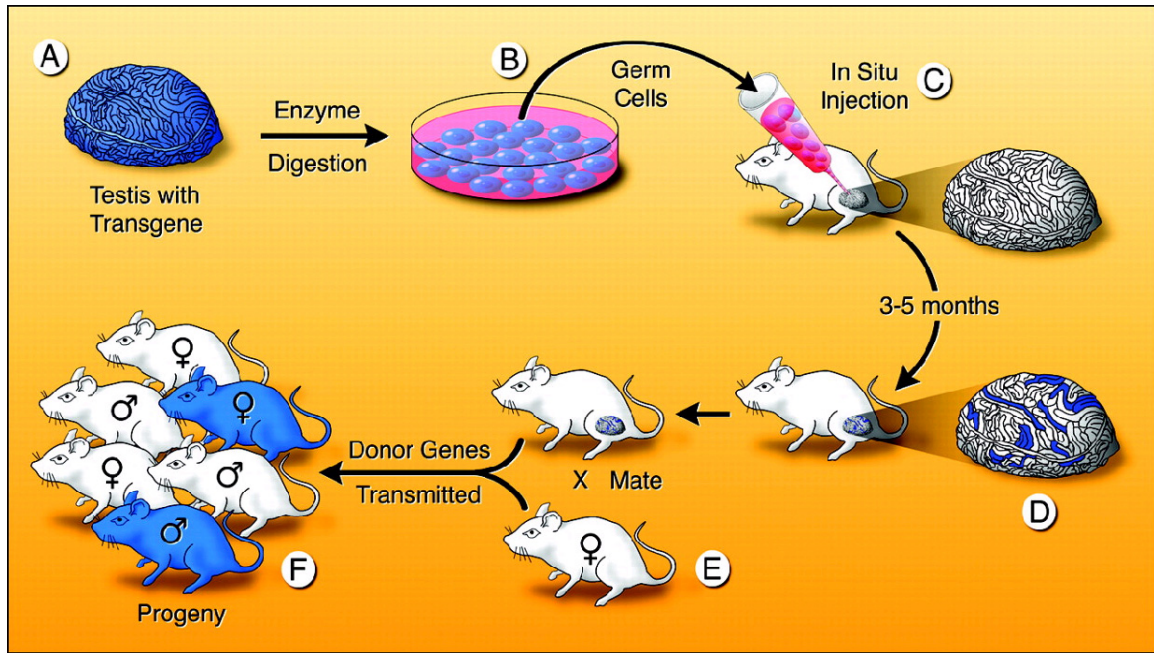
Figure 4. Testis cell transplantation method
Germ cells are acquired from a fertile donor testis (A). After culture (B) the cells are microinjected into the lumen of seminiferous tubules of a recipient mouse (C). Only spermatogonial stem cells can colonize the recipient testis. When the donor cells carry a transgene that stains the cell, colonies of donor cell-derived spermatogenesis are identified easily in the recipient testes (D). The recipient male can then be mated with a wild-type female (E) to produce progeny with the donor gene. Genetic modifications can be introduced while the stem cells are in culture (B). From Brinster, 2002.

The only established method to deliver transgenes directly into the male germ line is through retroviral-mediated gene delivery into spermatogonial stem cells in culture (see Fig. 4). Brinster and colleagues first reported successful retroviral infection of mouse and rat male germ stem cells *in vivo* or *in vitro* (Nagano et al., 2000; Nagano et al., 2002; Orwig et al., 2002a) and showed that transgenic mice could be formed carrying the retroviral transgene (Nagano et al., 2001). A transmission rate of about 4.5% was achieved and the transgene was transmitted to and expressed in subsequent generations. Transgenic rats also have been produced using cultures of germ stem cells that were transduced with a lentiviral EGFP-expression vector (Hamra et al., 2002). A single male rat transplanted with the transduced stem cells was able to transmit the viral transgene to approximately 30% of the total progeny and to 50% of the progeny derived from the cultured stem cells. Based on a transgene marking the cultures of donor cells (*MT-lacZ*) all pups inheriting the lentiviral transgene originated from the transplanted germ stem cells and were able to transmit both transgenes to subsequent generations.

1.4 Gene expression profiling

1.4.1 DNA microarrays

Microarray technology is a revolutionary tool in molecular research. Microarrays (Lockhart et al., 1996) and GeneChips® (Affymetrix Inc, Ca, USA (Pease et al., 1994)) are microscopic arrays of immobilized nucleic acids. Microarrays are robotically printed sets of PCR products or conventionally synthesized oligonucleotides, whereas Affymetrix GeneChips are high-density arrays of oligonucleotides synthesized *in silico* using light-directed chemistry. The use of microarrays extends to a wide range of analytical methods built around detection of sequence-specific nucleic acid hybridization. The technology can be used for gene discovery, disease diagnosis, drug discovery and toxicological research (The Chipping Forecast, 1999).

The most common application of microarray technology is transcript profiling. Microarrays make it possible to simultaneously detect differences in transcript abundance of a multitude of genes between two mRNA preparations. In a typical microarray experiment, two mRNA samples (e.g. a control and an experimental sample) are independently reverse transcribed into cDNA labeled with distinguishable fluorochromes, mixed and then hybridized to spotted microarrays (Fig. 5). Fluorescence intensities are measured for each gene, and ratios are computed. Typically, the magnitude of these ratios takes the form of a bell shaped curve, with a relatively small number of expression ratios exceeding a given threshold. In a typical GeneChip experiment, mRNA is converted into biotin-labeled antisense RNA (aRNA). The aRNA is fragmented, hybridized to the chip, and incubated with a streptavidin-conjugated fluorochrome. Signal intensities for all genes are quantified and can be compared to other samples.

In recent years there has been a dramatic growth in commercial sources of the components of the technology and in companies offering microarray services. Prices for commercially manufactured microarrays (Affymetrix, Agilent and various others) have come down significantly. Density and quality of annotation for microarrays have improved, making it possible now to analyze expression of almost the entire genome of complex organisms like human and mouse. For example, Affymetrix recently released new chips for mouse and human containing probe sets for more than 40,000 genes on a single chip. Increasing numbers of universities have established microarray facilities, making the technology available to a larger number of laboratories.
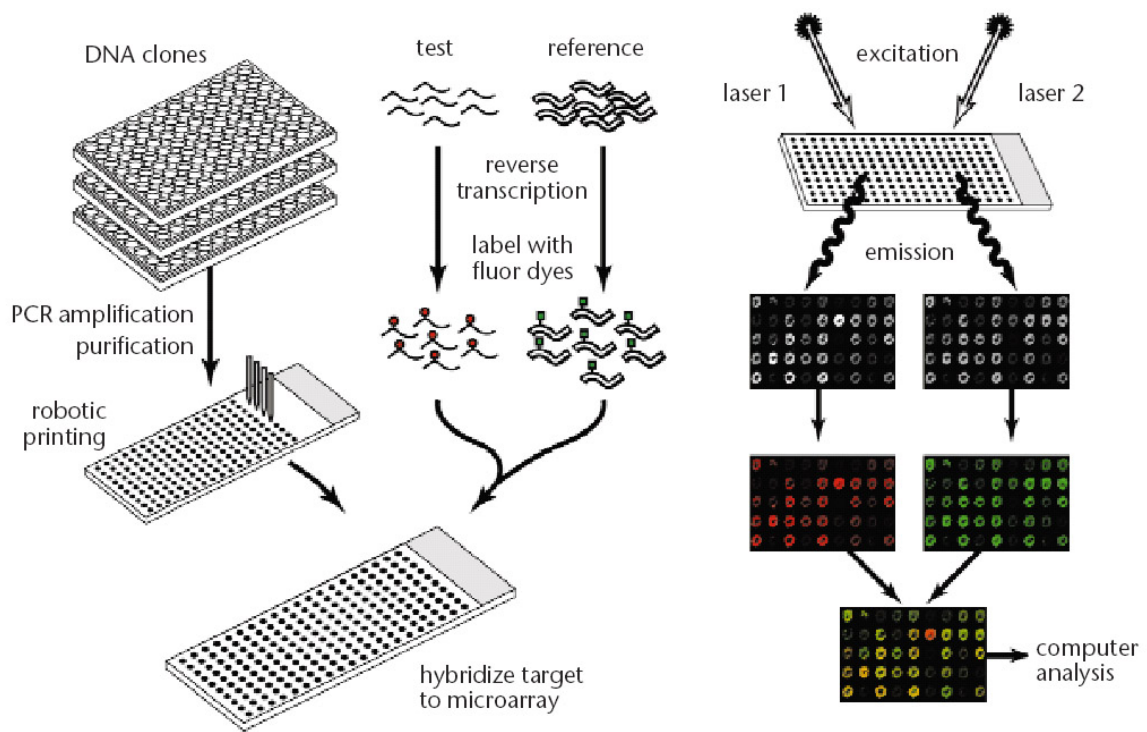
Figure 5. Generation and use of spotted cDNA microarrays
To generate microarrays, cDNA clones are amplified by PCR, the products purified and then robotically applied to glass slides. Two fluorescently labeled RNA or DNA samples can be hybridized to the microarray. After hybridization, fluorescence signals can be detected using laser excitation. The generated grey-scale images can be converted into pseudo-color images. Overlapping these color images produces the typical microarray images. From Duggan et al., 1999.

Compared to commercially available microarrays, chips generated in individual laboratories are often based on unsequenced cDNA libraries. It is ideal to know the sequence of all cDNA clones to be spotted, as this makes it possible to spot only one copy per gene and to thereby reduce redundancy completely. However, sequencing a large amount of clones is a very expensive and time-consuming task. For this reason a lot of researchers do not sequence their clones prior to spotting. These microarrays are often referred to as "discovery arrays" because specific clones are sequenced only after performing an experiment, if they show interesting changes in expression.

Sample labeling

The most typical technique to label a sample for microarray analysis is cDNA synthesis with direct incorporation of fluorescently labeled nucleotides. The fluorochromes of choice are usually the water-soluble cyanine dyes Cy3 and Cy5, as they offer distinct and non-overlapping emission spectra and therefore allow comparative analysis of two

samples on one microarray. However, there are some limitations to this approach. Cy-dye-modified nucleotides are bulky, and regular reverse transcription enzymes have a low affinity for them. This leads to a low labeling efficiency. The more important problem though is the uneven incorporation of the two different dyes. As Cy3 is smaller than Cy5, Cy3-modified nucleotides are incorporated at a higher rate than Cy5-modified nucleotides. The settings of a microarray scanner can be adjusted to partly compensate for the lower Cy5 signal. But with frequent occurrences of sequence-specific differences in incorporation, it is virtually impossible to eliminate the problem of uneven incorporation. It is therefore generally recommended to perform a dye-reversal experiment for each microarray slide and discard data for genes for which the separate experiments do not show matching results. Additionally, Cy5 is less stable than Cy3 and can easily be photochemically bleached by repeated exposure to the laser during scanning.

Amersham Biosciences (Piscataway, NJ) has tried to modify a reverse transcriptase and increase its affinity for Cy-dye modified nucleotides. Their 'Cyscribe' enzyme shows an improved incorporation rate, but we have performed experiments demonstrating that the increased incorporation rate is evened out by a lower yield of cDNA (data not shown).

An alternative to the direct incorporation of Cy-dye modified nucleotides is 'indirect incorporation'. This can be achieved in two steps: Incorporation of chemically reactive (amino allyl-modified) nucleotide analogs in a regular cDNA synthesis, followed by a coupling of reactive forms of Cy3- or Cy5-NHS esters in a separate reaction. This method leads to an even incorporation of Cy-dyes into individual samples. The small size of the amino allyl modified nucleotide also increases the incorporation rate.

Alternative labeling techniques have been developed and are gaining popularity. 'Direct labeling' is novel labeling methodology that uses platinum-linked cyanine dyes to directly chemically label mRNA (Gupta et al., 2003). The reagents are commercially available from Perkin Elmer (Wellesley, MA, Micromax Direct Labeling Kits). Genisphere (Hatfield, PA) has developed a labeling method which utilizes primers that are attached to a highly branched, fluorescently labeled DNA molecule ("dendrimer"), resulting in a high labeling efficiency of small transcripts in particular (Manduchi et al., 2002).

Another popular labeling technique is the generation of labeled aRNA through linear RNA amplification. Here, biotinylated UTP is incorporated into RNA in an *in vitro* transcription reaction. The biotin-UTP can then be labeled with a streptavidin-conjugated fluorescent dye. This labeling step is usually performed after hybridization of the biotinylated sample to the microarray. This method is generally used for all Affymetrix microarrays.

Molecular Probes (Eugene, OR) has developed a series of new fluorescent dyes, the Alexa Fluor dyes, some of which are now used for microarray experiments. Spectra of the Alexa Fluor 647 dye virtually match those of the Cy5 dye. Because of its increased brightness and photostability, Alexa Fluor 647 is a perfect candidate to replace the troublesome Cy5 dye.

Universal control samples

In a typical microarray experiment, two samples, labeled with different fluorescent dyes, are compared directly on one slide. This is a perfect situation for small experiments consisting only of two conditions, e.g. the comparison of a treated versus an untreated tissue culture dish or a transgenic animal versus a wild type. However, when working with a larger number of conditions, like a time course experiment, a different strategy has to be applied. Here every sample has to be compared to one control sample. Therefore, the same control sample has to be hybridized to every single slide. Ratios calculated from each microarray can then be compared to each other (the ratio of the ratios) to determine changes of gene expression between the individual samples. However, it is impossible to calculate a ratio for a gene for which the control channel has no signal. The ideal control sample therefore contains RNA for every gene on the microarray. For this purpose universal control RNA samples can be generated or purchased. These samples are prepared in large scale from a variety of tissues and provide a constant reference for all microarray hybridizations performed in a set of experiments (Puskas et al., 2002).

Affymetrix GeneChips

Ideally, the DNA spotted in each spot on the microarray is in excess of its respective labeled target. However, variability between spotted microarrays is usually so high, that a labeled sample yields different signals on separate microarrays. Performing dual-color hybridizations with the calculation of ratios avoids this problem. With low chip-to-chip variation though it is possible to hybridize only one sample to each microarray and then compare the intensities of each gene between microarrays. This approach eliminates all problems caused by the use of different dyes and eradicates the need for a universal reference sample. Large companies have the technology and can perform the quality controls needed to produce microarrays with low variability. Microarrays from Affymetrix and Amersham are generally used with single color hybridizations.

The Affymetrix chips have various advantages over their competitors' products: With hundreds of thousands of different oligonucleotide probes on each chip, each gene is represented by two sets of 25mer oligonucleotides: a set of eleven or more perfect

match oligonucleotides, and another set of an equivalent number of mismatch oligonu-cleotides (Fig. 6). In the mismatch oligonucleotides, one of the bases is substituted. Probesets with higher signal for the mismatch than the perfect match oligonucleotides are an indicator for unspecific binding, and data from these probesets can be discarded.
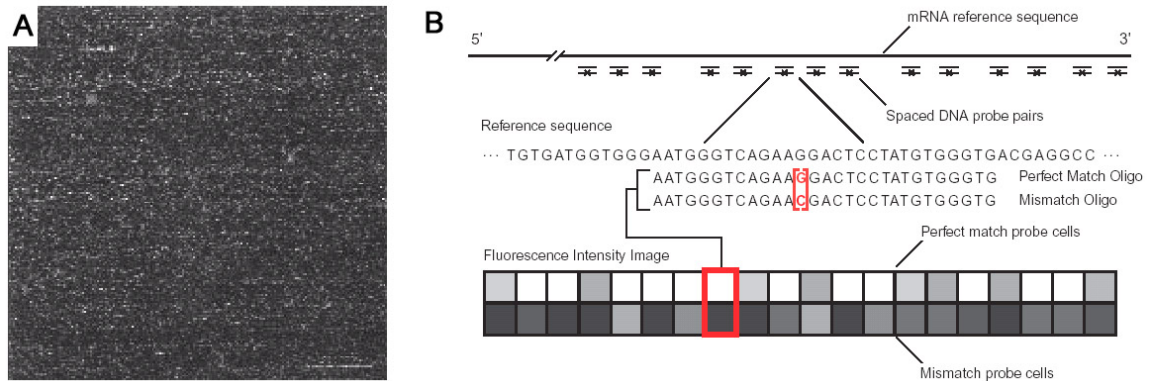

Figure 6. Gene expression profiling with Affymetrix oligonucleotide arrays
(A) A single 1.28 x 1.28 cm array contains probe sets for up to 40,000 genes and ESTs. (B) For eukaryotic organisms, probes are chosen typically from the 3´ end of the gene or transcript to re-duce problems that may arise from the use of partially degraded mRNA. The use of the perfect match and mismatch oligonucleotides across a set of probes greatly reduces the contribution of background and cross-hybridization and increases the quantitative accuracy and reproducibility of the measurements. Modified from Lipshutz et al., 1999.

GeneChip arrays have good sensitivity and show an extremely high reproducibility. They have a false chance rate of less than 2%, which is significantly lower than the potential variabilities derived from different biological samples (Nimgaonkar et al., 2003).

Detection limit and RNA amplification

The amount of available RNA can be a limiting factor in a microarray experiment and determines the labeling and detection methods (Fig. 7). Radioactive detection is very sensitive but is only suitable for low-density microarrays (macroarrays) because of its strong signals. Fluorescent labeling is the method of choice for high-density microarrays. While conventional tissue culture experiments typically yield sufficient amounts of RNA, certain experiments will only supply limited amounts. In order to perform microarray ex-periments on as little as a few nanograms of RNA, linear RNA amplification has to be performed. In this method, which was first developed to facilitate cloning of low-abun-dance messenger RNAs (Van Gelder et al., 1990), mRNA is reverse transcribed with an oligo-dT/T7 promoter primer. After synthesis of double stranded cDNA, antisense RNA is

transcribed *in vitro* by using a T7 RNA polymerase, generally resulting in a 1000-fold amplification of the original amount. If necessary, a second round of amplification can be performed simply by repeating the procedure. Several slightly different methods have evolved to produce higher yields and increase linearity (Baugh et al., 2001), but problems with variable transcript lengths remain (Luzzi et al., 2003).
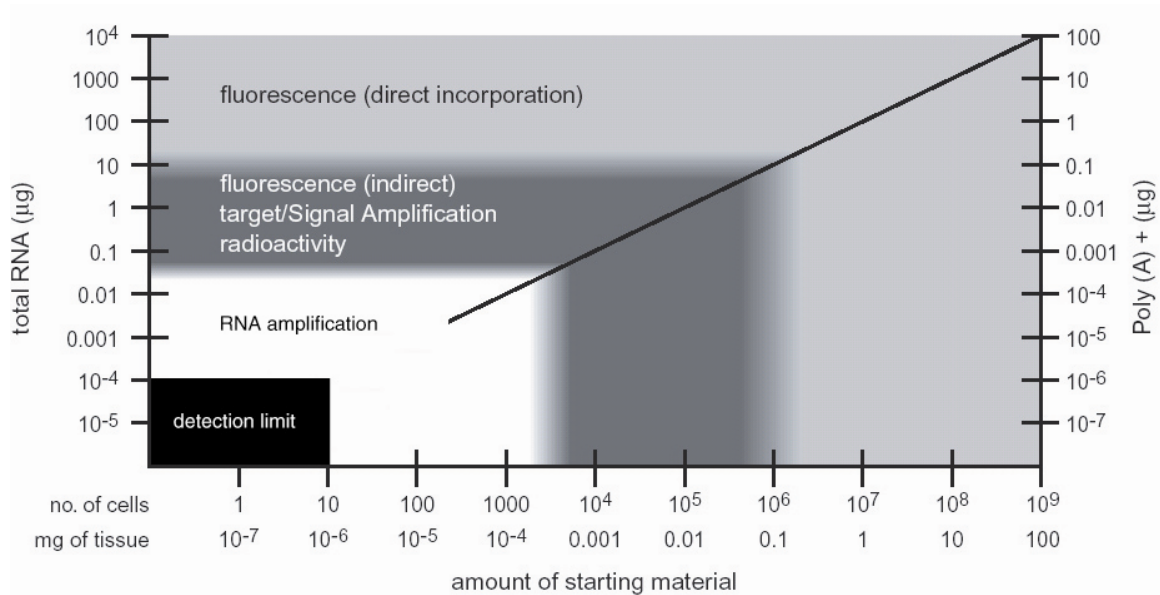

Figure 7. Detection limits using different labeling techniques
Depending on the biological material available, different labeling methods have to be used in order to obtain a detectable signal. The use of RNA amplification greatly reduces the detection limit of DNA microarrays. Modified from Duggan et al., 1999.

Microarray data analysis and publication guidelines

Data analysis is an extremely important aspect of every microarray experiment. Depending on the complexity of an experiment, different software tools are needed to allow the analysis of the vast amounts of data generated. While for a simple two-sample experiment the scanning software and Microsoft Excel are sufficient, larger experiments require specialized software for efficient data analysis. There is now a big supply of microarray analysis software packages. These tools facilitate ratio analysis, gene annotation, and sorting and filtering of genes. They perform statistical calculations and provide database connectivity. One of the most important functions of these tools is the ability to group genes according to their expression profiles, a feature known as cluster analysis. Clustering can be performed using a wide variety of algorithms (Slonim, 2002). The most common methods are hierarchical clustering, which arranges the data into a tree, and k-

means clustering, a method that partitions the data into a requested number of groups of genes with similar expression patterns. Choosing the clustering method is dependent on the data and the researcher's preferences. GeneSpring from Silicon Genetics, now in its sixth version, performs all the tasks described above, and the software has become one of the most popular tools for microarray analysis.

Since its invention, microarray technology has quickly spread around the globe and reached a large number of laboratories. Many different techniques have been developed and are constantly evolving. There are now countless publications including microarray data, and many significant results have been obtained through microarray experiments. However, until recently, there was no universal standard for presentation and exchange of microarray data. Numerous publications lacked precise descriptions of the microarray techniques used, and full data sets were only rarely made fully available to the public. In 2001, the Minimum Information About a Microarray Experiment (MIAME) standard was first proposed (Brazma et al., 2001). This proposal describes the minimum information required to ensure that microarray data can be easily interpreted and that results derived from its analysis can be independently verified. Compliance with this standard and sub-mission of the raw data to at least one public microarray database, like the Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/geo/) or ArrayExpress (www.ebi.ac.uk/arrayexpress), are now required by many journals.

1.4.2   Other techniques for global gene expression profiling

Microarray technology relies on the existence of specific cDNAs or knowledge of their sequences. Herein lies one of the shortcomings of this technology. Until the complete transcriptome of an organism is known, microarrays will not be able to distinguish changes in gene expression of all genes. Differential display and Serial analysis of gene expression (SAGE) are methods that do not rely on preexisting cDNA clones or se-quence information.

Differential display

The principle of differential display is the systematic amplification of the 3' termini of messenger RNAs by using anchored oligo-dT primers in combination with upstream ar-bitrary primers. The separation of the polymerase chain reaction products by gel electro-phoresis and their direct comparison allows the identification of differentially regulated genes (Bartlett, 2003). Recently, fluorescent differential display was established as the

first nonradioactive differential display system with equivalent sensitivity to the original ^{33}P isotopic labeling method. Because of its simplicity and sensitivity, differential display became one of the most widely used gene-screening methods in the early 1990s. However, differential display was quickly replaced by DNA microarrays, because of its lack of power, including a high number of false positives, and the failure to develop robust automated methods.

Serial analysis of gene expression

SAGE is a powerful tool that allows the analysis of overall gene expression patterns with digital analysis. Because this technique does not require preexisting clones, it can be used to identify and quantitate new genes as well as known genes (Velculescu et al., 1995). The basic concept of SAGE rests on two principles: Firstly, a small sequence of nucleotides from the transcript, a 'tag', can effectively identify the original transcript; secondly, linking these tags allows for rapid sequencing analysis of multiple transcripts. Having to sequence thousands of individual transcripts would take a long time to complete. By linking the tags together, only one sequencing event is required to sequence every transcript within the cell.

The biggest advantage of SAGE is its ability to identify rare and novel transcripts. However, because the tags generated during SAGE are extremely short (10-14 bp), there are problems with specificity, as certain tags are shared by multiple genes. The short length of the tags also makes it difficult to investigate the potential functions of unknown genes. Another problem is the variable length of the tags (Boheler and Stern, 2003).

1.4.3   Verification of expression changes of individual genes

The methods described above are designed for global gene expression analysis. There are several techniques that can be used to individually verify results obtained through global screens.

Northern blot analysis

In this technique, sample RNA is separated by denaturing agarose gel electrophoresis, then transferred to a solid support and immobilized. A radiolabeled or nonisotopically labeled RNA or DNA probe is then used to detect the message of interest. Typically, ethidium bromide-stained or radiolabeled RNA markers are run on the same gel as sample RNA to provide an accurate sizing ladder in gels or on autoradiograms.

Northern blotting is relatively easy to perform and only requires standard electrophoresis equipment; it not only gives information about the presence and relative abundance of a gene, but also about its size and the presence of alternatively spliced or multiple transcripts generated from a single locus. Northern blotting is also exceptionally versatile in the type of probe that can be used for hybridization. High specific activity random-primed or PCR-generated DNA probes, *in vitro* transcribed RNA probes, and oligonucleotide probes can all be used successfully.

The most important disadvantages of Northern blot analysis are its low sensitivity and problems with RNA degradation. To detect more than one message, it is usually necessary to strip the initial probe before hybridizing to a second probe. This process can be time consuming and problematic, although the use of strippable probes can simplify and improve this procedure.

Ribonuclease protection assays

The ribonuclease protection assay (RPA) is an extremely sensitive method for the detection and quantitation of specific RNAs in a complex mixture of total cellular RNA (Ma et al., 1996). The basis of the RPA is solution hybridization of an antisense probe (radiolabeled or nonisotopically labeled) to an RNA sample. After hybridization, single-stranded, unhybridized probe and RNA are degraded by ribonucleases. The remaining hybridized probe-target fragments are separated on an acrylamide gel and visualized by autoradiography. If nonisotopic probes are used, samples are visualized by transferring the gel to a membrane and performing a secondary detection step.

Since solution hybridization is far more efficient than filter-based hybridization and does not have the limitation of maximum membrane capacity, an optimized RPA may be 10 to 100 times as sensitive as Northern analysis and is much more tolerant of partially degraded RNA.

RPAs are the method of choice for simultaneous quantitation of several RNA targets. During solution hybridization and subsequent analysis, individual probe-target interactions are completely independent. Since location of signal is determined by the length of the homologous region of probe with target, several RNA targets and appropriate controls may be assayed simultaneously.

The primary limitation of RPAs is that they do not reveal information about message size. Protected fragment size is determined by the length of the homologous region of

probe with target - usually only 200-400 nucleotides. Another drawback to RPAs is that only antisense RNA probes can be used. In addition, the probe sequence must typically be completely homologous to the target (except for a small stretch of vector sequence at one or both ends of the probe). Therefore, partially related sequences (e.g., cross species or gene families) usually cannot be analyzed.

RT-PCR and real-time PCR

Reverse transcription followed by PCR (RT-PCR) can be used to detect gene expression differences between RNA samples. Because of the high sensitivity of PCR even rare messages can be detected. However, when using agarose gels to analyze PCR products, results are obtained from the end point of the reaction. The sample-to-sample variability of these endpoints severely limits the ability of RT-PCR to detect small changes in expression. Quantitative real-time PCR (qRT-PCR) provides a solution to this problem (Heid et al., 1996; Wilhelm and Pingoud, 2003). In qRT-PCR the accumulation of PCR product is measured during the PCR reaction. During the exponential phase exact doubling of product occurs with every cycle (assuming 100% reaction efficiency). However, the second phase of PCR, the linear phase, shows very high variability. Here, the reaction components are being consumed, the reaction is slowing, and products are starting to degrade. During the plateau phase (endpoint) the reaction has stopped, no more products are being made, and, if left long enough, the PCR products will degrade.

It is therefore ideal to measure PCR product accumulation during the exponential phase, before the reaction enters the highly variable linear phase. The qRT-PCR system is based on the detection and quantitation of a fluorescent reporter. This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during the exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. There are two general methods for the quantitative detection of the amplicon: fluorescent probes or DNA-binding agents. Fluorescent probes provide a gene-specific signal, as they are designed specifically for each gene. DNA-binding agents bind all double-stranded DNA generated in the PCR reaction and cannot distinguish between the desired PCR product and unwanted byproducts.

Real-time PCR assays are easy to perform, capable of high throughput, and can combine high sensitivity with reliable specificity, making the technique today's gold standard for the detection of gene expression differences.