Outlook

Emerging technologies for the molecular study of infertility, and potential clinical applications

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Abstract

The techniques currently used to treat infertility cases are quite limited in their capabilities, due to an incomplete understanding of the molecular activities of germ cells. Fortunately, several technologies are presently being researched that should aid in the understanding of the various molecular causes of germ cell pathologies. This review discusses microarray technology, proteomics, metabolic profiling, the PolScope, atomic force microscopy and microfluidics. These technologies have all seen success in preliminary studies, and promise directly or indirectly to improve the low success rates of IVF and other related therapies. However, their widespread use in laboratories and clinics may not be seen until preliminary studies confirming their safety and effectiveness are published, and until standardized protocols for their utilization are established.

Keywords: *infertility*, *IVF*, *microbiology*, *research*, *technology*

Introduction

It is estimated that worldwide, 15% of couples of reproductive age encounter fertility problems (Nishimune and Tanaka, 2006). This has been attributed to a variety of factors, including environmental pollutants (He *et al.*, 2006), and the increasing age at which couples decide to have children (Manipalviratn *et al.*, 2006). There are currently a number of techniques available to clinicians in diagnosing and managing infertility, but there are still limitations in the field. Regarding IVF, multiple pregnancies continue to be a problem (Manipalviratn, *et al.*, 2006), and success rates are greatly limited by the current embryo culture methods (Suh *et al.*, 2003). Fortunately, several technologies are presently being researched that should aid in the understanding of the various molecular causes of germ cell pathologies, thereby improving the clinical management of infertility cases.

Microarray

Despite detailed knowledge of testicular structure and accompanying cell biology, the current understanding of how this relates to the molecular pathways in the testis remains rudimentary. To gain a more detailed understanding of the molecular basis of male infertility, it would be advantageous to study the differences in gene expression between fertile and infertile males. If the differences in mRNA profiles, otherwise known as transcriptomes, are uncovered, there will be greater insight into identifying potential biochemical markers for infertility, as well as discovering clues to its indirect causes or direct triggers. Fortunately, the microarray is capable of analysing the transcriptome of cells and tissues (He *et al.*, 2006).

To learn more about the molecular events of spermatogenesis, researchers have used the microarray to compare the gene expression of spermatozoa from fertile and infertile men, and to compare the transcriptomes of germ cells at various stages in spermatogenesis. A number of novel genes associated with male fertility have even been identified using the microarray. As more research is conducted using this technology, it can be

anticipated that several molecular pathways associated with sperm physiology and pathology will be uncovered (He *et al.*, 2006).

The microarray also has potential for use in clinical diagnosis of male infertility. One large step in this direction was the creation of a spermatogenesis-related gene expression profile for human spermatozoa. This was paired with a demonstration that one could legitimately assess the overall health and quality of a sperm cell by analysing its gene expression signature using the microarray (Wang *et al.*, 2004).

To use this non-invasive technology to assess a male's fertility status, a semen sample is first collected, followed by isolation of spermatozoa from the ejaculate, and then isolation of mRNA from the spermatozoa. While functionally mature spermatozoa do not undergo transcription, they have been shown to contain mRNA, which was synthesized during spermatogenesis, providing insight of the specific spermatogenic events of an individual. The microarray is used to create an mRNA profile that can be compared with a physiologically normal gene expression profile for sperm. This would help to identify if the cause of infertility for a male is of spermatogenic origin. Utilizing the microarray to analyse a male's fertility status is sure to reveal much more information when compared with the techniques currently employed in clinics (Moldenhauer *et al.*, 2003).

There would be many advantages to creating microarray-based transcription profiles of oocytes at various stages of growth and maturation. It would result in a better understanding of the genes expressed during oocyte development, as well as those expressed at the various checkpoints that regulate the process. As of now, it is known that in a fully mature oocyte arrested in metaphase II (MII), the mRNA profile is essentially a collection of the mRNA transcripts created during its growth phase. In addition, disruption of transcription within an oocyte or modification of its current transcriptome could negatively effect its growth and development, as well as that of the embryo (Wood *et al.*, 2007).

Microarray analysis has indicated that the transcriptomes of MII oocytes of high morphological quality from normal women are distinctly different from the morphologically normal oocytes of women with polycystic ovaries (PCO). These molecular defects could help to reveal why women with PCO tend to have reduced fertility (Wood *et al.*, 2007). It has also been shown with microarrays that oocyte quality can be estimated based on the expression levels of at least 160 different genes (Zhang *et al.*, 2005). Many of these have been shown to be involved in pathways of cell growth and cell death (Inan *et al.*, 2006). There is great potential to find oocyte quality biomarkers among these transcripts, and gene expression levels of Pentraxin 3 (Ptx3), hyaluronic acid synthase 2 (HAS2), cyclooxygenase 2 (COX2; PTGS2) and gremlin (GREM1), have already been studied for this purpose (McKenzie *et al.*, 2004).

Studying the molecular processes involved in the development of a competent oocyte under ovarian stimulation conditions via microarray also has obvious advantages. Some studies have already discovered biomarkers associated with oocyte maturation using microarray (Gasca *et al.*, 2007). The information gained from these studies should ultimately help clinicians make more informed choices regarding the selection of ovarian stimulation protocols in female infertility cases. Such information could also lead to the selection of higher quality oocytes for IVF, and improve the culture and oocyte manipulation techniques that are used (Assou *et al.*, 2006). However, one must always keep in mind that even if a specific gene transcript is observed, not much information can be realistically known regarding the metabolic events within the cell. If microarray is to be utilized for studies of cellular activity, it is imperative that proteomics technology is also incorporated into the studies.

Proteomics technology

One complement to the field of gene expression analysis is proteomics (**Figure 1**). This is the study of protein abundance in cells or tissues, and involves the discovery of protein functions at the biochemical level (Rocken *et al.*, 2004).

Fundamental proteomic techniques include protein separation and the identification of proteins in biological samples. These can then be coupled to computational algorithms that allow the extraction of relevant information from the totality of data. Fortunately, several advances in proteomics, including the association of mass spectrometry with techniques of electrospray ionization (ESI) and matrix-associated laser desorption/ionization (MALDI), have greatly facilitated the ability to sequence and characterize peptides and proteins (Shankar *et al.*, 2005). Additionally, proteomics researchers are currently developing a method to compare protein profiles of cells and tissues in varied biological states. With such a development, it will be easier to learn more about the molecular activity and behaviour of cells (Rocken *et al.*, 2004).

One such development, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS), involves affinity-based mass spectrometry whereby proteins are absorbed to a chemically modified surface (e.g. cationic or anionic) or to a biochemical molecular surface (e.g. receptors or ligands). These different chip surfaces allow the various classes of proteins (hydrophobic, hydrophilic, acidic or basic) to be captured for analysis (Merchant and Weinberger, 2000). SELDI-TOF MS has been shown to capture, detect and analyse proteins directly from crude biological fluids. Alternatively, quadruple TOF (Q-TOF) identifies proteins by searching appropriate sequence databases. The high accuracy of the Q-TOF technology makes the hybridization of MALDI and Q-TOF a superior option for de-novo protein sequencing (Gygi and Aebersold, 2000).

Clinical proteomics is an emerging field that seeks to apply the science of proteomics in the search for biomarkers and the generation of protein profiles, which can help to predict, diagnose and monitor human pathologies (Verrills, 2006). One example of progress in this field was the utilization of SELDI-TOF MS in the discovery of biomarkers associated with normal cellular function for in-vivo mammalian embryonic development (Katz-Jaffe *et al.*, 2005). It has also been shown that there are significant alterations in the expression of proteins related to the morphological development of human blastocysts (Katz-Jaffe *et al.*, 2006). This new information has provided clinicians with an additional diagnostic tool when conducting IVF for infertile couples (Katz-Jaffe, *et al.*, 2005). Quantifying the potential

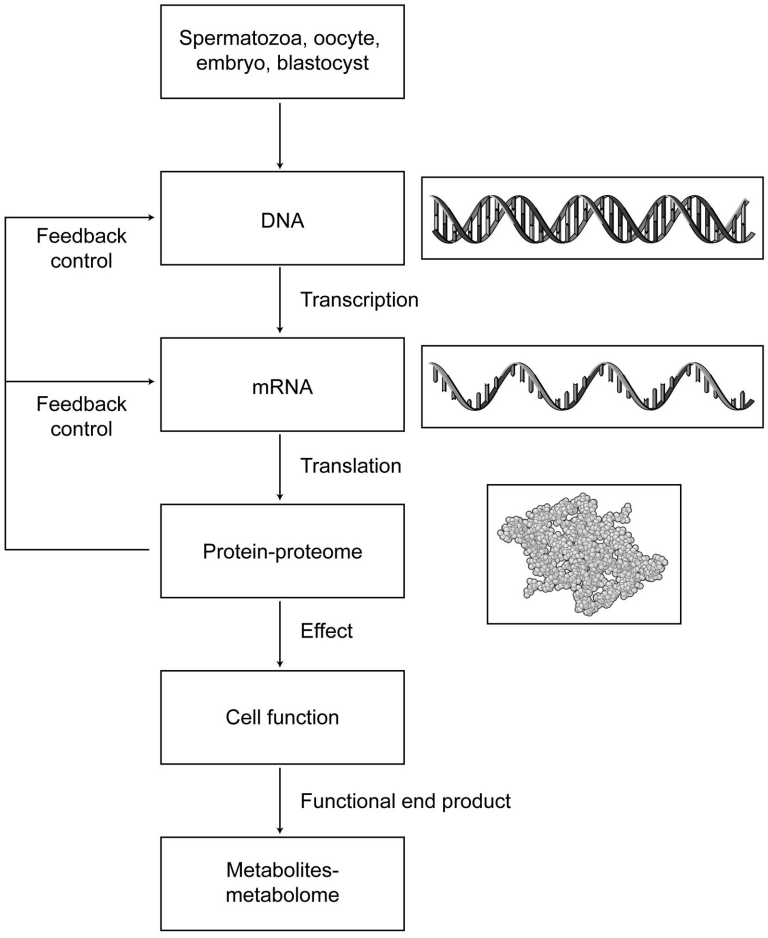

Figure 1. The flow of information at a relay from the genome to cellular metabolism. As per the 'central dogma' of molecular biology depicted in the figure, DNA is transcribed into RNA then translated to proteins, which then make small molecules. While there may be over 25,000 genes, 100,000–200,000 transcripts and up to 1,000,000 proteins, it is estimated that there may be as few as 2500 small molecules in the human metabolome.

viability of cultured embryos by non-invasive proteomic analysis should result in an increase in IVF pregnancy rates and live births, while allowing for a reduction in the number of embryos transferred, thus decreasing multiple gestations. Additionally, it has been found that infertile men have altered expression of at least 20 proteins. This is a clear indication that clinical proteomics will be an increasingly important asset to infertility researchers (Pixton *et al.*, 2004). Like microarrays, however, studying the proteome in isolation from mRNA and the resulting metabolites could easily lead to misinterpretation of the metabolic events occurring within cells. Researchers and clinicians must take this into account when considering the use of proteomics technology.

Metabolic profiling technology

Complimenting both transcriptomics and proteomics, metabolic profiling may also help unravel the complexity of disease state. Metabolic profiling, or metabolomics, is the analysis of various molecular metabolites within cells and fluids, and offers a significant advantage over the use of the two related fields of study. Since small changes in gene expression and protein synthesis result in an amplified change in metabolite profile, known as a metabolome, it is easier to detect subtle cellular events through the relatively new science of metabolomics (Deepinder *et al.*, 2007).

Several studies have been conducted which successfully attempted to use metabolomics to diagnose infertility and even help in assisted reproductive technology. However, in most cases, there is not yet enough quantitative information to use metabolic profiling for clinical applications. After further research and standardization, clinical metabolomics could be an invaluable tool for the infertility clinician (Deepinder *et al.*, 2007). It is estimated that the use of metabolomics may lead to: (i) more men seeking infertility evaluation; (ii) enhanced success rates in assisted reproduction; (iii) reduction in the

incidence of multiple births due to feasibility of single embryo transfer technique; and (iv) reduced health care costs associated with providing medical care to multiple premature infants (Deepinder *et al.*, 2007).

Individual metabolites often exhibit disturbances in concentration or flux during disease progression in an attempt to maintain cellular homeostasis in the organism. This can be measured using a variety of analytical methods. For example, when comparing normal males with those having various forms of male factor infertility, different levels of reactive oxygen species (ROS) were measured using a metabolomic approach (Agarwal, 2006). In addition, Thomas and co-workers used Fourier transform infrared spectroscopy (FT-IR) to analyse follicular fluids from large and small natural luteinized antral follicles. In-depth analysis of the spectra showed that inter- and intraspecific differences within the follicular fluids were not related to measured distributions of steroids, indicating that FT-IR could elucidate other aspects of follicular biochemistry that were important. It was proposed that the differences observed in the biochemical nature of the fluids may be reflective of the developmental capacity of the oocyte, suggesting that FT-IR could provide a biomarker related to oocyte quality (Thomas *et al.*, 2000).

With the development of highly sensitive non-invasive metabolic profiling techniques, it has been possible to attain a more comprehensive analysis of preimplantation embryos. It has also been beneficial in obtaining additional knowledge of the developmental physiology of embryos in general. This will facilitate the application of quantitative non-invasive methods of predicting developmental competence and viability of oocytes selected for use in IVF. For example, the oocyte selection procedure can start with the measurement of metabolites contained in follicular fluid (FF) which has been aspirated during oocyte retrieval. One study investigated the follicular fluid bone morphogenetic protein-15 (BMP-15) concentration and its relation to fertilization and embryo development. It was shown that high BMP-15 concentrations in FF are a positive indicator of good quality oocytes (Wu *et al.*, 2007). Such studies of non-invasive metabolomic profiling of embryo culture media may soon provide embryologists with a new tool for the accurate selection of viable embryos capable of producing a pregnancy. Additionally, this may potentially allow the future development of elective single embryo transfer programmes.

PolScope

The PolScope, a new type of polarized light microscope, facilitates visualization of the meiotic spindle in the live human oocyte without damaging the cell. Based on the results of several studies, higher fertilization rates were obtained among oocytes in which a morphologically normal spindle was present, and the lack of a normal spindle was found to be an indicator of abnormal chromosome alignment (Cohen *et al.*, 2004). Hence, the use of the Polscope allows for a more thorough oocyte quality assessment before sperm injection for IVF (Moon *et al.*, 2003).

Chromosome misalignment can lead to an aneuploid embryo, and would result from the natural ageing process or from oocyte micromanipulation during IVF. Since aneuploid embryos can develop to the blastocyst stage, there is always a chance that a transferred embryo is aneuploid. The use of the PolScope to directly observe the spindle allows clinicians to avoid injection of oocytes that have failed to fully organize their meiotic spindle and have not reached full meiotic maturity. This should ultimately result in greater IVF pregnancy rates (Wang *et al.*, 2001). In addition to spindle imaging, non-invasive analysis of other oocyte morphological parameters, such as zona morphology or polar organization, should help to improve treatment protocols for individual patients.

Atomic force microscopy

The recent application of atomic force microscopy (AFM) to study germ cell pathophysiology will greatly benefit infertility researchers. However, its potential role as a diagnostic tool for clinicians remains to be established. AFM provides nanometer resolution, topographic data images of the natural surface structure of biological samples, and shows potential as a novel three-dimensional (3D) image contrast device (Joshi *et al.*, 2001a). Topographic images are captured by scanning the AFM tip over the sample and recording the *z*-axis displacement required to maintain a constant contact force (Binnig *et al.*, 1986). The nanometer resolution of the AFM imaging technique has been used to examine the structural features of proteins, producing data that is comparable to that obtained by X-ray crystallography (Muller *et al.*, 1995). However, the biggest promise of the AFM technique is in the measurement of the activity of single protein structures *in vivo* during signalling events or during other types of cellular activities. One study demonstrated that it is possible to observe conformational changes in the structure of a single nuclear pore in *Xenopus* oocyte nuclear envelopes (Perez-Terzic *et al.*, 1996). However, these measurements were done in fixed tissue. Shortly after, plasma membrane structures were shown undergoing conformational changes during cellular activity using AFM (Schneider *et al.*, 1997).

Spermatozoa have been shown to be good subjects for imaging with AFM because of their rigidity and relatively small size. Moreover, in contrast to the artificial preservatives and elaborate sample preparation used for electron microscopy (EM), only simple preparation of samples, such as air-drying of smeared spermatozoa, is required for AFM observation. Because AFM allows the direct observation of spermatozoa in their native surroundings, analysis of their submolecular structure and activity becomes an exciting possibility (Kumar *et al.*, 2005).

Morphological alterations in spermatozoa from semen samples with oligoasthenoteratozoospermia and asthenozoospermia have been analysed using AFM (Joshi *et al.*, 2001a). This study clearly indicates that morphological alterations can be the result of infection, and extensive information on such changes involving the head, neck and flagellum are provided. Similar studies have shown other dimensional changes in sperms from patients with a varicocele (Joshi *et al.*, 2001b).

Many other important discoveries in the field of fertility research have been made with AFM. In one study, high resolution AFM imaging was performed on uncoated, unfixed and unstained sperm chromatin. Surface images of the intact

chromatin revealed large nodular subunits ranging from 50 to 100 nm in diameter, thought to be one level of packaging above the protamine–DNA complex. It was also shown that the nodules were arranged along thick fibres that spread out from the nucleus upon decondensation (Allen *et al.*, 1993).

It should be understood, though, that one can obtain more precise information about the molecular abnormalities of sperm cells when AFM is paired with other complementary observational techniques such as immunocytochemistry (Chemes and Rawe, 2003).

Microfluidics

Current research in assisted reproduction is striving to achieve in-vitro culture conditions that mimic the in-vivo micro-environment. Microfluidics, a recent technology, shows promise as an alternative for each step of the IVF process. The microfluidics system is constructed on a microscale and offers the potential of embryo culture in exceedingly small volumes, but with flow of the culture medium (Suh *et al.*, 2003). It consists of a system of channels in the micrometer range that is fabricated by liquid-phase photopolymerization, lithography and laminar flow. It can be constructed to contain actuators, valves and sensors using the same construction platform (Beebe *et al.*, 2000). Additionally, the use of microfluidics improves DNA and protein analysis procedures by reducing the cost and time of such procedures, as well as the size of biological samples (Barry and Ivanov, 2004). Because this nano-level platform allows the analysis of very small quantities of samples and reagents, it is capable of revealing much molecular information regarding germ cell pathologies (Khademhosseini *et al.*, 2006).

The use of a system of microfluidics offers several advantages over the current standard IVF protocol. While several improvements must be made before the design is approved for use, microfluidics can provide a simple way to isolate motile spermatozoa of relatively normal morphology from good quality or poor quality semen. This method is found to reduce the time and labour involved in sperm isolation, as well as decrease the amount of ROS produced in the process, yielding better quality spermatozoa (Schuster *et al.*, 2003). Additionally, it was postulated that maturing oocytes for IVF in a microfluidic system would yield better quality embryos, since it more closely resembles in-vivo conditions than current in-vitro techniques (Beebe *et al.*, 2002)

Current embryo culture conditions for IVF are not considered very natural, since they utilize one culture medium from insemination until embryo transfer, whereas embryos produced *in vivo* are bathed in an environment whose fluid contents are always in flux. In fact, it has been shown that the environmental conditions that support the embryo at one stage of development can be harmful if the embryo is exposed to those conditions later in the maturation process (Gardner *et al.*, 1998). Because microfluidics allows gradual change of culture medium contents, greater IVF success rates can be expected. It also significantly reduces the amount of manual embryo manipulation, which is potentially damaging to the cell (Suh *et al.*, 2003).

The use of intracytoplasmic sperm injection (ICSI) has produced much ethical debate, since the mechanisms of natural selection are bypassed. However, it has been the only means available to allow males with severe oligospermia to have success with IVF. Microfluidics offers a potential alternative for such patients, since only 100 spermatozoa are required for this new IVF process, which does not bypass natural selection. This is because coincubation of the oocyte and spermatozoa takes place within a volume of 1 µl, hence the spermatozoa are sufficiently concentrated to facilitate fertilization (Suh *et al.*, 2003).

Ultimately, a complete microfluidics system may be established, where each step of the current IVF process is replaced by a single automated 'IVF-laboratory-on-a-chip,' requiring minimal cell manipulation (Beebe *et al.*, 2002). This is indeed a realistic future goal, but it will require that more studies and tests be performed to establish optimal operating conditions for each step of the IVF process, followed by their standardization. Additionally, the difficulty of fabricating and packaging such technology is proving to be a significant obstacle in promoting the widespread use of microfluidics. Only after these issues are resolved can an integrated 'IVF-laboratory-on-a-chip' be implemented as a legitimate IVF technique (Suh, *et al.*, 2003).

Conclusion

The aforementioned technologies show great potential to unveil the molecular causes, effects, and indicators of infertility. Therefore, one can expect the low success rates of IVF and other related therapies to increase over time. However, these technologies can only be legitimately utilized, once they are proven effective in their function, and their use is well standardized.

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