Molecular aspects of male fertility

International Workshop on Molecular Andrology

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Introduction

Two crucial challenges face modern andrology: to find a solution for male infertility and to develop an effective, reversible male contraceptive. Infertility is estimated to affect at least one in 20 men

worldwide, and the majority of cases are of idiopathic—possibly genetic or epigenetic—aetiology. Technical advances in assisted reproduction, such as the intracytoplasmic injection of defective sperm or immature germ cells into a mature egg, have transformed the treatment of male infertility; however, new concerns have been raised about the propagation of genetic or epigenetic abnormalities that might otherwise be excluded by normal fertilization or conventional *in vitro* fertilization. In addition, andrology is at the epicentre of the stem cell revolution, as spermatogonial stem cells (SSCs) have become a crucial model system to explore stem cell potential. It is hoped that the understanding of the regulation of SSCs will allow the restoration of spermatogenesis in infertile men. The need to overcome these diverse challenges and seize emerging opportunities is driving andrologists to decipher the molecular pathways—which are often altered in disease—that govern the male reproductive system.

Against this dynamically evolving background, many of the world's leading experts in andrology gathered at the Altes Schloss in Giessen for the 5th International Workshop on Molecular Andrology, which was also the first Symposium of the newly established Clinical Research Unit in Andrology at Marburg and Giessen. The castle, which is used as a museum and art gallery, was host to 90 excellent scientific presentations and the surrounding discussions, organized around three main topics. The first topic was the regulation of differentiation and self-renewal of SSCs and the use of these cells as a source of pluripotent stem cells. The underlying nuclear organization, epigenetics and imprinting of SSC differentiation were discussed in the second set of talks, in which novel structures and mechanisms that regulate inheritance were presented. Finally, the third topic concerned the function of the somatic Sertoli cell within the immune-privileged environment of the testis, with presentations providing unique insights into the crucial role of these cells in enabling spermatogenesis and their potential roles in effective gene therapy. Owing to space limitations, this report highlights the main findings rather than providing a comprehensive overview. We apologize at the outset for all of the exciting work we could not include.

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Glossary

BrdU	bromodeoxyuridine
CCL	CC chemokine ligand
c-Src	cellular homologue of the viral Rous sarcoma oncogene
CTCF	CCCTC-binding factor
ETV5	ets variant gene 5, a transcription factor
GFP	green fluorescent protein
H2AL	histone cluster 1, H2AL
IL	interleukin
JAM-A	junctional adhesion molecule A
LINE	long interspersed nuclear element
<i>Oct4</i>	octamer-4, a transcription factor
OVA	ovalbumin
RNAi	RNA interference
SCID	severe combined immunodeficiency
SINE	short interspersed nuclear element
TEX14	testis expressed 14, a testis protein implicated in intercellular bridge formation
TGF β	transforming growth factor β
VEGF	vascular endothelial growth factor
XX ^y	mouse strain carrying two X chromosomes and one mutated Y chromosome
ZO-1	zonula occludens-1

The testis and its development

The testis comprises two interacting compartments: the seminiferous tubules and the interstitial tissue. Haploid spermatozoa arise from SSCs at the periphery of the tubules, following a complex schedule of proliferation and differentiation (Fig 1). Epithelial Sertoli cells provide support but are dependent on interactions with the peritubular cells, steroidogenic Leydig cells and other cells of the interstitium. The traditionally accepted idea that seminiferous cord formation is promoted by peritubular cells that migrate from the mesonephros during development has recently been challenged. It is becoming clear that the migrating cells are in fact endothelial cells (Cool *et al*, 2008; Combes *et al*, 2009), a point which B. Capel (Durham, NC, USA) demonstrated clearly in a movie showing GFP-labelled endothelial cells that migrate out of the mesonephros to the testis along specific tracks. The cells in the interstitium produce VEGF, which attracts the endothelial cells. In turn, the endothelial cells could elicit Notch signalling, which is required to maintain Leydig progenitors. When either VEGF or Notch signalling is altered, tubule formation is severely disrupted, thereby affecting fertility.

During development, primordial germ cells proliferate and migrate to the genital ridges, where they are incorporated into the seminiferous cords as gonocytes. Mouse gonocytes proliferate until approximately E15-16 and then become quiescent until after birth; the molecular factors that mediate this growth inhibition are unknown. S. Moreno (Paris, France) showed that TGF β 2 inhibits the proliferation of gonocytes in E13.5 mouse testes in culture and prolongs quiescence in neonatal testes. Mice with conditional deletions of the type II TGF β receptor in primordial germ cells and their progeny had more gonocytes at E13.5 than wild-type mice. In addition, in E13.5 cultured type II TGF β receptor knockout testes, gonocyte proliferation was prolonged compared to normal testes. These results confirm another important role for TGF β signalling in the regulation of primordial germ cell and gonocyte proliferation.

Spermatogonial stem cell differentiation

In rodents, SSCs are considered to be single cells, and the first sign of differentiation is the formation of a pair of daughter cells connected by an intercellular bridge that functions as a channel connecting the germ cells. In mammals, intercellular bridges exist between differentiating spermatogonia up to the spermatozoal stage and TEX14 is required for their formation (Greenbaum *et al*, 2006). Interestingly, some spermatocytes still form in *Tex14*^{-/-} testes, as described by M. Matzuk (Houston, TX, USA), indicating that SSC differentiation is not absolutely dependent on the presence of the intercellular bridges. S. Schlatt (Münster, Germany) described the state of the art with respect to SSC transplantation, a technique that enables the SSC capacity of germ cell suspensions to be studied. SSC transplantation is being applied in an increasing number of species and aspects of SSC investigation.

Many studies have contributed to our understanding of rodent SSCs and spermatogonia morphology and differentiation kinetics. The SSC niche has recently been identified within the regions of the seminiferous tubules that are adjacent to larger areas of interstitial tissue. At this workshop, R. Nóbrega (Utrecht, The Netherlands) introduced the zebrafish as a promising new model for the study of SSCs. He showed the existence of long-term BrdU-retaining germ cells, which are putative stem cells, in zebrafish testes. As in rodents, these cells are preferentially located close to the interstitial tissue, which suggests that the location of SSCs near interstitial tissue could be a general principle among vertebrates. D. De Rooij (Amsterdam, The Netherlands) used the wealth of rodent data available to develop a computer model which simulates the behaviour of SSCs during the epithelial cycle. According to this model, SSCs undergo symmetrical divisions and either one or both daughter cells migrate out of the niche areas and differentiate, whereas daughter cells of other SSCs stay in the niche, thereby maintaining the SSC pool. The model is successful in predicting the numbers of SSCs and spermatogonial cell pairs and chains of varying lengths that are generated during more than 50 epithelial cycles, which are in agreement with those seen in Chinese hamster tubules. The model is also consistent with data obtained in ageing mice (Nakagawa *et al*, 2007) and can account for the low efficiency of SSC transplantation.

The transcription factor ETV5 is essential for mouse male fertility. M. Hofmann (Urbana, IL, USA) showed that in *Etv5*-deficient mice, there is already an absence of germ cells in some seminiferous tubules during the first wave of spermatogenesis, indicating a failure in the maintenance of SSCs. Interestingly, the production of chemokines of the CCL family—notably CCL9—by Sertoli cells was also reduced. These chemokines were found to strongly affect SSC migration, and could even be responsible for the uniform distribution of SSCs over the basal membrane area.

W. Engel (Göttingen, Germany) provided an overview of the state of the art in the derivation of pluripotent stem cells from SSCs. His group compared the transcriptome of multipotent adult germline stem cells before and after these cells had undergone some of the first steps of differentiation. Twelve genes were identified as important pluripotency candidates. One of these, appropriately named gene 12, seemed to be testis-specific and act upstream of *Oct4*. This is a notable development, as it might be the long-sought molecule that could be used as a marker of SSCs and a gene that is crucial for the regulation of SSC differentiation potential. The characterization of gene 12 is ongoing.

Fig 1 | Spermatogenesis and the organization of the sperm genome. The seminiferous tubules within the testis provide a well-defined system to characterize stem cell differentiation. Interstitial cells, most notably the steroidogenic Leydig cells, are distributed throughout the space between the tubules. Each tubule is surrounded by a basement membrane and layer of peritubular cells, and the epithelium comprises the developing germ cells and supporting Sertoli cells. Note the progression of germ cell differentiation from the basement membrane region, where the SSCs and spermatogonia are positioned, followed by meiotic reduction divisions of the spermatocytes that produce the haploid gametes (spermatids), and the release of the spermatozoa into the lumen. As the germ cells divide and differentiate, they remain connected by intercellular bridges that allow the exchange of cytoplasmic and other components. During differentiation, the germ cells are subject to a range of epigenetic modifications, including dynamic histone modifications and restructuring of the entire nucleus. Most of the nucleosomal histones are replaced by a series of testis-specific histone variants, which are in turn replaced by various transition proteins and finally by protamines. This replacement compacts the paternal genome to one-thirteenth of the size of the oocyte genome and divides it into histone-enriched and protamine-enriched compartments, which are organized by the nuclear matrix. RNAs are distributed throughout the nuclear matrix. The entire spermatozoa is delivered to the oocyte at fertilization. In addition to the centrosome and the paternal genome, RNAs and other components probably contribute to the birth and future well-being of a healthy child. Figure adapted from Krawetz (2005) and <http://bio1152.nicerweb.com/Locked/media/ch46/spermatogenesis.html>. SSCs, spermatogonial stem cells.

Germ cell epigenomics: from organization to expression

The session on epigenomics was introduced by S.A. Krawetz (Detroit, MI, USA), who presented an overview of the changes that occur in nuclear organization during spermatogenesis (Fig 1). These changes are characterized by the replacement of the majority of histones with protamines and the formation of an almost crystalline structure. Interestingly, the remaining histones segregate to the nuclear annulus, suggesting a differential compartmentalization of the chromatin. Changes in gene expression also occur during this process, and Krawetz discussed a new mechanism of gene activation initiated by topoisomerase, a component of the nuclear matrix that specifically nicks the genome to mark and delimit the boundary of an open chromatin domain. The spermatozoal nuclear matrix seems to store various RNAs, including mRNAs that are currently used to diagnose

infertility (Lalancette *et al*, 2009) and small non-coding RNAs, both of which have been postulated to have a crucial role in the development or the onset of adult disease. As T. Karr (Tempe, AZ, USA) emphasized, the protein composition of sperm is unique; the complexity of the *Drosophila* sperm proteome seems limited—381 proteins including histone variants—which is comparable to that observed in humans and presented by R. Oliva (Barcelona, Spain) but less complex than the mouse sperm proteome. The application of emerging technologies, including the refined separation of tryptic peptides by immobilized pH gradient strips and nano-scale two-dimensional liquid chromatography followed by electron spray ionization mass spectroscopy, should help to resolve this conundrum.

S. Rousseaux (Grenoble, France) initiated a discussion of male-specific epigenome lineage specification. The majority of histones

exist primarily in a hypoacetylated state until the spermatid stage, when global hyperacetylation occurs within the elongating spermatid; signalling is then elicited to replace the majority of the histones with protamines. As the spermatids condense, the acetylated histones are generally removed, but the pericentromeres remain bound to histones enriched with H2AL1 and H2AL2. This binding is apparently mediated by a testis-specific double bromodomain-containing factor (Rousseaux *et al*, 2009). A global analysis of histone replacement in mice and humans was presented by D. Miller (Leeds, UK). The degree of histone replacement (85–98%) varies between species, fuelling a debate regarding the need for the remaining histones for species survival. The histone and protamine compartments can be isolated by differential salt extraction or micrococcal digestion and Miller's group characterized them by array comparative genomic hybridization. Of note, olfactory genes and zinc finger genes were enriched in the protamine-bound fraction. By contrast, promoters of paternal responsive genes required for early embryonic development were marked by acetylated histones in close proximity to CTCF binding sites in hypomethylated regions (Arpanahi *et al*, 2009).

The rest of the session focused on clinical outcomes and mechanisms of disease. E. Rajpert-De Meyts (Copenhagen, Denmark) discussed the contribution of environmental endocrine disruption, which affects KIT/p53/Oct4, and of arresting gonocyte differentiation or promoting dedifferentiation of the spermatogonia, to the onset of germ cell neoplasias in young males. J. Wistuba (Muenster, Germany) described the initial characterization of a Klinefelter (karyotype 41, XX^y) mouse line that exhibited hypergonadotropic hypogonadism, germ cell loss and cognitive deficits, and should provide a valuable model system to study the epigenetic nature of this disease. M. Hou (Giessen, Germany) addressed the question of biobanking male germ cells before the treatment of childhood leukemia with Velcade® or similar agents, to give male patients the opportunity to pass on their genetic heritage. A vigorous discussion followed regarding assisted reproductive technology, as 4–10% of children conceived through these methods have medical problems, some of which are likely to be associated with defects in imprinting. As noted by N. El Hajj (Mainz, Germany), this could be due to a range of paternal effect epimutations, such as abnormal SINE and LINE repeat methylation, which are exacerbated in oligospermia. Although pregnancy rates are unaffected, a higher rate of miscarriage is observed after assisted reproduction. Whether the initiating event that leads to epimutations is random or whether a given mechanism or epigenetic mediators, such as RNAs, have a role in this process is still unclear.

The Sertoli cell supports and protects spermatogenesis

The maintenance of an environment that is appropriate for the development of germ cells and protects them from toxins, infection and host immune responses is vital to maintaining fertility. The Sertoli cells of the seminiferous epithelium are primarily responsible for providing this support and protection by maintaining the blood–testis barrier (BTB), recognizing and reacting to pathogens and controlling local immune cells (Fig 2).

An intact BTB maintains the unique physiological environment required by postmeiotic germ cells and contributes to their protection from infection and autoimmunity by restricting the movement of molecules and cells into the tubules. The main structural components of the BTB are the junctional complexes that are

established between adjacent Sertoli cells, comprising tight junctions, gap junctions, basal ectoplasmic specializations and desmosome-like junctions (Xia *et al*, 2005). These structures and their regulation are considered highly promising targets for contraceptive drugs. D. Mruk (New York, NY, USA) elegantly showed that the RNAi knockdown of the desmosome proteins desmoglein-2 and desmocollin-2 in Sertoli cells *in vitro* is sufficient to disrupt barrier function. Her group has discovered a novel regulatory protein complex—composed of desmoglein-2, the crucial junctional proteins ZO-1 and JAM-A, the coxsackie and adenovirus receptor, and c-Src—that is an essential part of the BTB.

The protective role of the BTB is supported by specific immunoregulatory mechanisms. For example, Sertoli cells have unique immunological properties that provide graft protection in co-transplantation studies (Dufour *et al*, 2003). When pancreatic islets and Sertoli cells are co-transplanted into mice, the number of Sertoli cells transplanted is critical for graft survival, as shown by J. Dufour (Lubbock, TX, USA). Furthermore, graft survival depends on factors that are secreted by the Sertoli cells, rather than on the reconstitution of the structural features that are characteristic of the seminiferous epithelium, such as the BTB. Dufour also showed that Sertoli cells can be engineered and used to deliver therapeutic proteins. Her group injected transduced Sertoli cells containing a modified human insulin gene into diabetic SCID mice, which led to insulin production and reduced blood glucose levels. Although these observations are exciting, the mechanisms responsible for Sertoli cell immunoregulation need to be elucidated before they can be applied. K. Tung (Charlottesville, VA, USA) provided a glimpse into the complexity of the mechanisms involved, using two BALB/c mouse lines that express a 40-fold different concentration of transgenic OVA as a surrogate autoantigen in elongated spermatids. In the mice that express high amounts of OVA, the antigen was detected within the residual bodies that are cast off by the sperm at the time of their release, and in immune complexes within the interstitial tissue. These mice were resistant to immune responses or orchitis after active immunization with exogenous OVA. By contrast, the antigen was not detectable in the mice that expressed low amounts of OVA; they developed OVA-specific T-cell and antibody responses and severe autoimmune orchitis after immunization. These data provide the first evidence that antigen exposure can create antigen-specific tolerance in the testis, possibly under the influence of Sertoli cell immunoregulation.

M. Hedger (Melbourne, Australia) showed that similar immune-related mechanisms underlie the response of Sertoli cells to infection and inflammation in addition to providing normal spermatogenic supporting functions. Even in the absence of infection, Sertoli cells produce a distinctive pattern of immunoregulatory cytokines under the influence of the adjacent germ cells and residual bodies (O'Bryan & Hedger, 2008). These cytokines, which include IL1 α , IL6 and activin A, exert direct effects on the development and proliferation of spermatogonia and spermatocytes, and they modulate the response of Sertoli cells to the follicle-stimulating hormone, which is their principal trophic hormone. The regulation of these cytokines, in both the normal and inflamed testis, could involve the engagement of pattern-recognition receptors—which are responsible primarily for the recognition of microbial pathogens—such as the Toll-like receptors and the signalling pathways they elicit. Thus, it seems that these cytokines mediate critical regulatory processes

Fig 2 | Sertoli cell signalling protects and supports spermatogenesis. Highly specialized junctional complexes between adjacent Sertoli cells create a blood–testis barrier that prevents direct communication between all but the earliest germ cells (spermatogonia) and the interstitial tissue and vasculature. This communication is instead mediated through the Sertoli cells, which produce cytokines in response to the presence of developing germ cells and also in response to inflammation and infection. A role for pattern recognition receptors (PRRs)—expressed either on the Sertoli cell membrane or in the cytoplasm—has been implicated in this communication process. The cytokines produced by the Sertoli cells regulate spermatogonial and spermatocyte development, junctional integrity and the function of immunoregulatory cells present in the interstitium (dendritic cells and resident macrophages). Germ cell antigens, present in the residual bodies that are cast off when sperm are released or from degenerating germ cells, traverse the basement membrane to be taken up by antigen-presenting dendritic cells in the interstitium, leading to antigen-specific tolerance.

that might be overridden by the activation of the Sertoli cell during inflammation, which thereby contributes to the damage caused by inflammation and infection.

Summary

This workshop highlighted many important advances in our understanding of the differentiation of SSCs and spermatogonia and its regulation. Notably, we are closer to understanding the complex molecular factors that are involved in the establishment and maintenance of the SSC niche, which might be harnessed to provide a true source of multipotent stem cells. Although significant progress has been made towards characterizing the cells of the spermatogenic lineage, from chromatin structure to RNA expression to disease, we have really only scratched the epigenetic surface. Our knowledge of the ordered nature of the packaging of sperm chromatin has increased, but remains insufficient to understand how higher-ordered structures regulate the expression of the human genome, thereby influencing subsequent embryonic development. A role for both large and small RNAs in regulating genome stability and evolution was suggested at this meeting and we are sure that many surprises lie ahead in this area of research; today, we are still left to ponder their effects on disease. The

exposure to foreign compounds can markedly affect fertility, as elegantly illustrated during the course of the meeting, perhaps by inducing paternal-effect epimutations that could be exacerbated through the use of assisted reproduction technology. The study of how Sertoli cells provide germ cell support and protection through their physical and immunoregulatory functions should yield important insights for both andrology and immunoregulation, potentially facilitating the transfer of gene therapy from the bench to the bedside.

Finally, it should be emphasized that the many unique features of the spermatogenic process—ranging from the complexities of the spermatogonial niche, the unique features of sperm chromatin repackaging and the epigenetic state, to crucial aspects of local immune regulation—make this a fascinating and productive model for the study of many molecular aspects of cell development, genetics and epigenetics.

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