Journal of Andrology, Vol. 26, No. 6, November/December 2005

Synopsis: XVIIIth Testis Workshop, "Testicular Cell Dynamics and Endocrine Signaling"

The Testis Workshop convened in Seattle, Wash, from March 30 to April 2 at the Grand Hyatt Hotel. The theme for 2005 was ''Testicular Cell Dynamics and Endocrine Signaling.'' Recent years have brought an influx of new information into the field of male reproduction. Several laboratories have been able to apply the genomics approach to gene expression in the male, revealing previously unknown patterns of gene expression and gene products that were localized in male reproductive tract tissues and cells for the first time. These discoveries paved the way for the next wave, an opportunity to analyze male reproductive biology and the processes by which sperm are formed in the seminiferous tubule and by which androgen is synthesized in the interstitium of the testis. The different levels of organization in the testis, including the stages of spermatogenesis, the enzymatic steps of steroidogenesis, and the intracellular signaling pathways of hormones, are now more amenable for study and selection of potential targets for drug development. The 2005 workshop was organized into 6 sessions: Genetics; Development; Endocrine Axis; Steroidogenesis; Spermatogenesis; and Clinical Correlates. Each of the 2 full days of the meeting also had 2 short (10-minute) oral talks that were selected from among the submitted abstracts and 2 integral poster sessions. These infused late-breaking developments into the program.

The theme of the meeting was immediately engaged in the Keynote Lecture delivered by **Ryuzo Yanagimachi** (University of Hawaii). The topic of his talk was paternal contributions to early embryogenesis that are brought into the oocyte by the sperm during fertilization. Dr Yanagimachi's perspective on sperm biology spans over 40 years, culminating in the technical feat of cloning mice, ironically bypassing the involvment of the sperm and using somatic cell nuclei to initiate embryonic development in enucleated oocytes. Dr Yanagimachi facetiously raised the question of whether there is any remaining role for the male as the new era of reproductive cloning dawns. He hastened to add that problems with epigenetic errors that have yet to be overcome afflict cloned animals. Moreover, sperm will remain vitally necessary for reproduction because sexual recombination increases genetic diversity, ensuring the continued fitness of the species in the face of environmental change. Dr Yanagimachi then reviewed the cell-to-cell interactions between sperm and egg, including the mechanisms of egg activation and block to polyspermy. The haploid male nucleus is the most critical component of the sperm to be assumed into the egg. Structural and genetic defects can lead to abortive development of the embryo. Embryos resulting from fertilization by sperm with chromosomal abnormalities are usually aborted before term. At the end of his talk, Dr Yanagimachi took a few moments to reminisce about his mentors and the events that drove him to take up science as a career. Ever anxious to understand the complexities of fertilization in a variety of species, he planned a collaborative visit to the Japanese island of Hokkaido to study fish sperm.

The first full day of the workshop began with a Benchmark Lecture given by **Luis Parada** (University of Texas Southwestern Medical Center, Dallas) on the molecular genetics of early sexual differentiation in the male. In particular, the role of insulin 3 (INSL3, also known as relaxin-like factor) in transabdominal descent of the testis was examined. The mouse knockout of the INSL3 gene has cryptorchid testes and this is the only phenotype observed in the male. Leydig cells appear to be the exclusive sites of INSL3 expression. Working from the observation the xenoestrogens increase the frequency of cryptorchidism, Dr Parada studied the regulation of INSL3 by estradiol and found that it was down-regulated in Leydig cells. To further explore the pathways of insulin signaling that are required for testis descent, a triple knockout of the insulin receptor, insulin-like growth factor 1 receptor, and insulin receptor–related receptor was generated. Each by itself did not fully replicate the cryptorchid phenotype obtained by the INSL3 knockout: this only occurred when all 3 were targeted together.

Three platform presentations followed in a session devoted to development. Richard Behringer (MD Anderson) explained the regulation of the Mullerian Inhibiting Substance (MIS), which causes the regression of the anlagen of female reproductive structures. Through the use of conditional knockout mouse lines, it was demonstrated that MIS acts by the Wnt signaling pathway to increase beta-catenin. **Michael Skinner** (Washington State University) presented findings showing a multigenerational effect of in utero exposures to the environmental xenoestrogen methoxychlor. In male rats, increased testicular cell apoptosis and decreased fertility resulted from these exposures,

DOI: 10.2164/jandrol.05120

which could be explained as an epigenetic problem. Skinner concluded by showing evidence of abnormal DNA methylation in several genes that might influence testis function. **Niels Geijsen** (Massachusetts General) reported the differentiation of embryonic stem (ES) cells into male germ cells. Dr Giejsen and colleagues have developed an in vitro culture system in which ES cells form embryoid bodies, in which subsets of cells segregate into the spermatogenic lineage as embryonic germ cells, and can be induced to self-renew when stimulated by retinoic acid. It is also apparent that these embryonic germ cells undergo erasure of imprinting of specific sets of genes normally, such as occurs normally in vivo. More mature male germ cells can be derived from the embryonic germ cells and they have been shown to partially support development when microinjected into oocytes. The potential of this research to alleviate male infertility was discussed.

The male endocrine axis was set as the topic of the workshop's Thursday afternoon session, and **David Puett** (University of Georgia) was the first of 3 speakers. He presented a structure analysis of the luteinizing hormone receptor (LHR). A 7-transmembrane G-protein–coupled receptor, the LHR is chiefly expressed in Leydig cells where, upon binding to LH, it induces synthesis and secretion of testosterone. The key events to be understood from a biochemical perspective are ligand binding and receptor activation, and Dr Puett's primary studies made use of site-directed mutagenesis, protein engineering, and molecular modeling. Among other findings, he was able to show that reorientation of transmembrane helices in the LHR is associated with mutations resulting in constitutive activity. **Leslie Heckert** (University of Kansas) has recently investigated the function of the steroidogenic factor 1 (SF-1), an orphan nuclear transcription factor that acts at the earliest stages of male sexual differentiation, regulating development of the pituitary, adrenal, and testis. Studies of SF-1 function in vivo have been hampered by the fact that targeted deletion of the gene in mice is lethal shortly after birth. Dr Heckert generated several yeast artificial chromosome constructs that contained portions of the rat SF-1 gene. Lines of SF-1 knockout mice containing these constructs had partial restoration of SF-1 function. One line of hypomorphs was created expressing a truncated SF-1, resulting in deficient Leydig cell development and androgen status, revealing the importance of SF-1 signaling in this cell type. **Phillipa Saunders** (University of Edinburgh) analyzed the distributions of the various forms of the estrogen receptor (ER) in the male reproductive system. The forms include ERα and ERβ, with the β form having 3 splice variants in the human. The functional significance of ERβ expression in the testis will continue to be studied, no doubt, as a result of the evidence of disruptive effects of xenoestrogens on the testis, which occur preferentially through β rather than α. The ERβ2 variant protein is truncated and does not bind steroid. Since ERβ2 is not expressed in rodents, it is unclear whether results from rodent models of estrogen action can be simply extended to humans.

On Friday, the morning began with the second Benchmark Lecture delivered by **Walter Miller** (University of California, San Francisco), who reviewed the enzymatic steps and accessory proteins that together enable cells to synthesize steroids. His talk was subdivided into 3 portions: a review of the role of steroidogenic acute regulatory protein (StAR) in mediating cholesterol transfer to the mitochondrial inner membrane; 17α-hydroxylase; and human mutations in genes encoding steroidogenic enzymes producing disorders of steroid synthesis. StAR now appears to operate primarily at the surface of the mitochondrion rather than as a channel or protein shuttle within the membranes. Phosphorylation of 17α-hydroxylase is required for enzymatic activity. The flavoprotein P450 oxidoreductase (POR) donates the electron necessary for NADPH-activated 17α-hydroxylase catalytic activity. The POR gene has been knocked out in mice, and there is a naturally occurring loss-of-function mutation producing Antley-Bixler Syndrome in humans (manifested by skeletal anomalies).

Three platform presentations followed in the session on steroidogenesis. **Peter O'Shaughnessy** (University of Glasgow) has studied the development of both fetal and adult Leydig cells in the mouse. Using high-throughput screening of messenger RNAs, several genes were seen to be expressed in fetal but not adult Leydig cells, and the genes were expressed in a pronounced developmental pattern. One gene of interest encodes the melanocortin type 2 receptor, the receptor for adrenocorticotrophic hormone (ACTH). Accordingly, ACTH stimulated steroidogenesis in fetal Leydig cells. The results may indicate a neural ontogeny of the Leydig cell lineage. **Buck Hales** (University of Illinois, Chicago) returned to the cholesterol mobilization function of the StAR protein in Leydig cells, but with greater emphasis on the role of the mitochondrion as an active mediator. Leydig cell mitochondria maintain an electrochemical gradient, which is essential for the internalization of the processed form of StAR. Dissipation of the gradient blocks both StAR internalization and steroid synthesis. Further studies showed that calcium flux, cellular adenosine triphosphate, and active mitochondrial respiration are each implicated in maintaining steroid synthesis. **William Duax** (Hauptman-Woodward Medical Research Institute, Buffalo, NY) closed the session energetically, showing how physical chemical principles can be used to make structure function predictions for enzymes in the steroid dehydrogenase family. He presented a technique developed from bioinformatics that identifies ''fingerprints'' of 30–40 amino acid residue stretches as common elements in many of

the proteins in this family, which can be used reliably to predict parameters such as cofactor preference and substrate specificity.

Friday's afternoon session covered spermatogenesis. **Tony Plant** (University of Pittsburgh) investigated the pubertal onset of spermatogenesis in rhesus monkeys. There is an extended hypogonadotropic phase in monkeys during which the testis is spermatogenically quiescent. However, stem spermatogonia continue to proliferate, and their mitoses are therefore independent of gonadotropin. Expression of the KiSS-1 protein in the hypothalamus ends the juvenile phase, stimulating a resurgence of gonadotropin-releasing hormone release. Dr Plant has used osmotic minipumps to prematurely increase LH or follicle stimulating hormone (FSH) in juvenile monkeys. LH rapidly elicits mature levels of testosterone production, but 4 to 6 months of FSH stimulation are required for initiation of spermatogenesis. **Clint MacDonald** (Texas Tech University) studied polyadenylation of messenger RNAs in germ cells. Polyadenylation, a stretch of about 250 adenyls at the 3′ end, is a means of conferring message stability and thereby increasing the half-life of the mRNA. Male germ cells have differences in polyadenylation compared to somatic cells. Dr MacDonald discovered a protein, τCstF-64, a testis-specific form of the protein that mediates polyadenylation in somatic cells. The CstF-64 somatic form is an RNA binding protein encoded by a gene on the X chromosome. A knockout of the *Cstf2t* gene that encodes τCstF-64 results in male sterility. **Kate Loveland** (Monash University, Melbourne) is interested in the role of transforming growth factor β (TGFβ) superfamily signaling in germ cell maturation. She focused her talk on activin, which uses Smads as intracellular mediators that move into the nucleus to modulate transcription. In general, TGFβ cytokines signal through Smads 1, 5, and 8. Activin, however, uses Smad 2, but not Smad 3. Examination of activin knockout mice led to the discovery of a class of importin proteins in the testis that are essential to nuclear localization of transcription factors.

The final session of the workshop (on Saturday morning) was given the title ''Clinical Correlates''; this portion of the workshop looked to the future of research leading from basic to translational. **John McCarrey** (University of Texas, San Antonio) provided a panoramic summary of epigenetic regulation of germ cells in the last Benchmark Lecture. As a model to follow regulatory events, Dr McCarrey analyzed the testis-specific form of the housekeeping enzyme phosphoglycerate kinase 2 (*Pgk2*). A footprinting assay of DNase 1 hypersensitive sites revealed that there were protein interactions with a CAAT box and Sp-1 site in the *Pgk2* promoter sequence. Dr McCarrey then explained the histone code, by which the configuration of the DNA is made more or less open for transcriptional activity, which was evaluated using chromatin immunoprecipitation assays. Demethylation of *Pgk2* occurs during the spermatogonial stage, which is consistent with the timing of PGK2 expression. A knockout of *Pgk2* was achieved in collaboration with Mitch Eddy's lab. Two platform presentations followed. The first, by **Jon Jarrow** (Johns Hopkins Medical School), showed the results of measuring androgen concentrations in small samples of intratesticular fluid obtained via minimally invasive puncture-aspiration biopsies of human testes. It was necessary to develop precise analytical chemistry to detect steroids, given the small sample volumes obtained. Therefore, the analysis was conducted using tandem liquid chromatography mass spectrometry. Consideration was given to estimating total and unbound androgen through measurement of sex hormone binding globulin and androgen binding protein. Based on these data, Dr Jarrow concluded that other additional binding proteins may be present in human testis. This method now makes it possible to define the relationship between intratesticular androgen level and rate of sperm production in the human. The second talk was given by **Benjamin Kaupp** (Helmholtz Research Center, Juelich, Germany) and concerned chemotaxis of sea urchin sperm. Calcium channels open on the flagellum in response to a cyclic guanosine monophosphate (GMP)-mediated signal induced by the chemoattractant to change the flagellar wave. Turning activity of swimming sperm was monitored with a stroboscopic ultraviolet light apparatus.

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