Asian Journal of Andrology (2015) 17, 764–766

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INVITED COMMENTARY

Sperm Biology

Looking both ways: new research on old theories

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Asian Journal of Andrology (2015) **17**, 764–766; doi: 10.4103/1008-682X.160265; published online: 24 July 2015

In the 22 years since its inception this meeting[1] has become the focus of a still small but growing and devoted group of scientists, meeting purposefully at a leisurely interval of four years, so that only substantial advances are reported. As these meetings approach a quarter of a century of epididymal research, it is time to reflect on the focus of former, and direction of future, epididymal research. Over this time, there has been a marked change from the initial physiological studies that highlighted epididymal function in terms of of sperm responses to external influences, to the prevalent highly technical and complex biochemical and biophysical procedures applied to epididymis, its main regions, its epithelia and individual cells *in vivo*, *in vitro* and *in silico*, in attempts to explain the ability of the organ to influence spermatozoa throughout their posttesticular existence. These are usually linked to histo- and cyto-chemical studies of fixed, or increasingly of live, organs, tissues and cells, made possible by advances in fluorescence microscopical techniques, and real-time computerized analysis of huge datasets.

The introductory lectures, from currently retired senior investigators, were to set the scene by glancing backwards and establishing the *raison d'ĕtre* of the meeting where current research would be presented. **Mike Bedford**[2] thoroughly discussed the progress of his early animal epididymal research and introduced the evidence leading to the concept of sperm maturation, and the roles of androgens and heat on sperm storage in the epididymis. The human epididymis, while supporting maturation, has a low storage capacity, and a limited ability to sustain spermatozoa. This introductory talk provided participants with knowledge of many of the biological processes that their research is directed toward. **Trevor Cooper's** talk[3] first appraised the beauty of the epididymis, from the older purely anatomical techniques of luminal injection and corrosion casts of vasculature, and more recent fluorescence techniques revealing the various epithelial cell types with cytoplasmic extensions to the lumen of cells of intra-epithelial and extra-tubular origin. He tried to rationalize current knowledge with the more nebulous concepts of sperm maturation and storage, but sensed a mismatch between the overwhelming amount of data on epididymal secretions and their regulation, and the scant knowledge of how they may act to modify spermatozoa during their maturation. Nevertheless, he was optimistic enough to suggest that we now know sufficient to be able to test a system *in vitro* to mimic the process of sperm maturation. **Terry Turner**[4] provided a talk different from all the others at the meeting, with an emphasis more on the scientist than the science. He examined what triggers people to enter science, and why they stay in it. Likening the thrill of scientific discovery to that which an early European discoverer must have sensed when, expecting a land mass, he first glimpsed instead the Pacific Ocean, he discussed the "Balboa moments" in science: the times of excitement when an unexpected discovery spurs one to more exploration and discovery. One of his such moments was that despite its three macroscopical anatomical divisions and 10 histological, connective tissue-defined segments, the mouse epididymis showed unexpectedly six major clusters of gene activity. The location of gene expression did not correspond to the histological segments, as had been speculated from his own work on the possibly restrictive barriers to growth factors of connective tissue septa, but rather overlapped them. Taking a clue from this reappraisal of current knowledge, he also urged us to continue to challenge dogma.

In the remaining meeting, work was presented on sperm proteins that undergo changes during the maturation process. **Mark Baker's** work[5] on the fertility-related testicular sperm protein IZUMO1 showed posttranslational modification involving glycosylation, which, when prevented by point mutation, leads to subfertility, with parallel changes to the protein's location on the equatorial segment, as a sperm-egg interaction interface. Its phosphorylation is related to a testis-specific kinase, and its degradation is dipeptidase-related. The receptors for IZUMO1 on eggs are CD9 and Juno, of which Juno is shed postfusion, an event preventing polyspermy. To date, the protein has not yet been related to human infertility. **Paty Cuasnicú**[6] summed up the history of the early-discovered secreted epididymal protein known then as protein D/E, but now as a member of the CRISP family. Various CRISP proteins are produced in the epididymis, with Crisp1 being involved in capacitation, sperm-zona binding and sperm-egg fusion. Crisp1 is both loosely attached by ionic forces and dependent on concentrations of zinc, when it may function as a decapacitation factor, and as a tightly attached GPI-anchored form that moves to the equatorial segment upon the acrosome reaction, when it may be involved in egg interactions via its N terminal 12 amino acids. A relationship to fertility has been shown by mouse knockouts of Crisp1 (leading to reduced egg interaction if cumulus-free, but not in-cumulus, eggs are used) and of Crisp4 (the males are fertile but their spermatozoa exhibit lower binding to the zona pellucida). Immunization of male monkeys with an antihuman CRISP1 leads to reversible infertility. **Julia Dorin**[7] presented her lab's. work on the large family of β-defensins; antimicrobials which interact with immune cells and which are present at high expression in the epididymis. These proteins are involved in sperm maturation since selective deletion of a cluster of defensin genes in mice produces a low siring rate, but infertility after several generations of back-crossing. In the latter defensin cluster-free animals, epididymal histology and sperm numbers appeared normal, but the spermatozoa showed reduced motility, precocious capacitation, spontaneous acrosome reactions, and an inability to bind to oocytes. Flagellar fragility indicated by decapitated spermatozoa confirmed a major role of defensins in male fertility. Human infertility may also reflect defensin abnormalities since men with a frame-shift mutation in DEFB126 have low paternity, and infertile men with low sperm motility have low amounts of sperm human β-defensin 1.

Several papers presented new data on the forms of epididymosomes and the modes of transfer of their cargo to spermatozoa. **Patricia Martin-DeLeon**[8] has continued her study of hydrophobic, GPI-linked proteins of epididymal origin on the sperm surface by demonstrating transfer by vesicular and nonvesicular routes. Membrane-bound epididymosomes dock on the sperm surface whereas lipid carriers in the membrane-free fluid fractions mediate direct insertion; the latter transfer process being more efficient.

She demonstrated that the transfer from epididymosomes of transmembrane proteins, *e.g.,* PMCA4, a plasma membrane calcium transporter Ca^{2+}-ATPase responsible for calcium efflux from spermatozoa, requires docking of epididymosomes to the sperm membrane before fusion mediated by CD9. **Robert Sullivan**[9] updated his work on two population of epididymosomes involved in the transfer of different epididymal proteins both to live and dead spermatozoa. Transfer to live spermatozoa, considered a mechanism of sperm maturation, involves cholesterol-rich vesicles with the membrane raft marker CD9 and other proteins including P25b, and the transfer of such proteins is inhibited by antibodies to tetraspanins. Another epididymosomal population, characterized by the interaction of ELSPBP1 with the phospholipid head group choline, transfers proteins (including HE12, CE12) to dead or dying spermatozoa, where the transfer is [Zn^{2+}]-related. As the ELSPBP1 partner is biliverdin reductase A, which reduces biliverdin to bilirubin (both antioxidants), these proteins most likely prevent dead cell-related oxidative damage from affecting live cells. He emphasized the selective uptake of proteins from epididymosomes onto spermatozoa, as only a subset is transferred, confirmed by the different proteomes of epididymosomes in different epididymal regions, and he considered the possibility of fusion of epididymosomes with spermatozoa, allowing the vesicle's cytoplasmic protein cargo to the sperm interior. **Clemence Belleannée**[10] addressed the role of these epididymal microvesicles in the transfer of extra-cellular microRNAs (miRNAs), released from one epithelial cell type into epididymal and seminal fluid, and interacting with migrating spermatozoa as well as downstream epithelial cells. She described membrane-derived exosomes, endosomes containing multivesicular cargo released during apocrine secretion, and how their miRNA cargo is transferred to recipient cells after endocytosis or membrane fusion. These new observations on the influence of regulatory miRNAs raise the possibility of dysfunction of these processes causing infertility, but also permitting noninvasive diagnosis of infertility.

Two papers covered topics closely related to those above. **Petra Sipilä**[11] discussed the various roles of the RNAse III enzyme Dicer 1, currently accepted as important for cleaving pre-miRNA to miRNAs. Recently, miRNA-independent functions for it have been discovered, including the production of small interfering RNAs, and its binding to messenger RNA and long noncoding RNAs. Although Dicer1 is not required for epididymal development, prepubertal conditional knockout of Dicer1 in the proximal epididymis causes de-differentiation of the epithelium, defects in lipid homeostasis, and unbalanced steroid receptors. This leads to male infertility, but unlike that in other initial segment-free knockout epididymides, resulting not from sperm swelling but from sperm motility and flagellar fragility. **Joël Drevet**[12] continued the story of sterols in sperm membranes, hypercholesterolemia, and male infertility. While cholesterol levels can change many organs, including the testis, his review concentrated on the epididymal-specific effects of hypercholesterolemia. The epididymal-related fertility repercussions of dyslipidemia can be explained by changes upon maturation in sperm membrane cholesterol, the phospholipid/cholesterol ratio and the cholesterol-rich micro-domain membrane lipid rafts. The latter organize cell signalling and dictate which proteins enter the membrane from epididymosomes. Knockouts of the transcription factors LXRa/b, which are activated by oxidized forms of cholesterol, gives rise to infertile adult males, but the young have no phenotype unless given a high cholesterol diet when epididymal pathologies become evident. These include epididymal epithelial morphological changes ("foamy" cells) and sperm damage: decreased motility, viability, and weak axonemal structures. This other side of the epididymal protein-sperm interaction highlights the complex role that epididymal fluid must play in both the provision of suitably posttranslationally modified proteins, and the optimal manipulation of the sperm membrane lipid bilayer that permits uptake of relevant exogenous proteins to the fertility-associated sperm domain.

Two papers addressed epididymal structure and function. **Barry Hinton**[13] presented the knockout mouse models with abnormalities of the development of the Wolffian duct and mesonephros. He discussed their importance for understanding such development and how they contribute to understanding human clinical abnormalities of the tract such as CBAVD. In a thorough review of the multifarious events resulting in mesonephros formation, tubule elongation and coiling, regional and cellular differentiation, the factors regulating these transitions (transcription factors and their receptors involved in epithelial-mesenchymal transition; secreted glycoproteins and their roles in cell proliferation and polarity; luminal and circulating factors responsible for stabilizing the coiling and elongation and coiling) were listed, as were the animal models of abnormal development of the Wolffian duct.

In a clinically-relevant presentation, **Meinhardt**[14] discussed the segmentation and regional variation in immunoregulatory genes and pathogen-detection systems in the epididymis, especially the possible conflicts arising from the dual roles of the innate immune system in controlling infection and providing the immuno-tolerance required to prevent the generation of auto-antibodies against spermatozoa. Although acute ascending epididymitis normally responds to antibiotic treatment, azoospermia may persist as a result of compromise of the system creating immuno-tolerance for spermatozoa when severe inflammatory responses damage the epididymis. He went on to discuss the etiology, symptoms, diagnosis, management, clinical course and impact on infertility of epididymitis from animal models.

The articles in this Special Issue, and presentations at the meeting undocumented here, serve to show that Cooper's worries of their being more details ("warp") than comprehension ("weft"),[3] most likely due to a retiree's failure to keep up with the field, were unfounded, since many of the articles reviewed above directly presented evidence about the nature of sperm-fluid interactions[6,8–10,12] or revealed how these changes affect sperm structure and function.[7,11,12] They also show that for whatever reason, deficiencies in epididymal knowledge can be rectified by such Epididymal Conference meetings.

Several similar observations were made by groups using different animal models: (1) the zinc-dependence of protein binding to both live[6] and dead[9] sperm cells; (2) the possibility of fusion of epididymosomes with spermatozoa, allowing the direct transfer of membrane proteins,[8] and the cytoplasmic transfer of proteins[9] and miRNA[10] to spermatozoa; (3) the fragility of maturing spermatozoa in mice lacking proteins (β-defensins),[7] an enzyme (Dicer1)[11] and transcription factors (LXRa/b);[12] (4) the importance of lipid homeostasis and the role of membrane lipids in determining protein uptake and sperm function revealed by dietary lipid changes[12] or gene knockout.[11] Whether these similarities stem from the same underlying defect or are convergent developments, remains to be established and presented at Epid VII, which should take place in Montréal Canada in 2018.

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Afterword to the 6th International Conference on the Epididymis, Shanghai special issue in the Asian Journal of Andrology.