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THE LOCALIZATION OF MULTIPLE CATHEPSIN mRNAs IN THE SEMINIFEROUS EPITHELIUM BY *IN SITU* HYBRIDIZATION IS CONSISTENT WITH THEIR ROLE IN GERM CELL(GC) MIGRATION. ((Sanny S.W. Chung[1,2], Li-ji Zhu[1], Meng-yun Mo[1], Will M. Lee[2] and C. Yan Cheng[1].)) [1]The Population Council, 1230 York Avenue, New York, New York 10021; and [2]Department of Zoology, The University of Hong Kong, Hong Kong, Hong Kong.

Proteases play a crucial role in GC migration throughout spermatogenesis. Previous studies by RT-PCR have shown that Sertoli as well as Leydig cells express the mRNAs of cathepsin B, C, D, H, L and S while GC isolated from adult rat testes express all of the above cathepsins except cathepsin D. However, GC, in particular spermatocytes and spermatogonia, isolated from immature rat testes at 10-20 days of age also expressed cathepsin D. These results suggest an active involvement of GC in their migration. To examine if GC indeed express multiple cathepsin mRNAs, we have localized the mRNAs of cathepsin D, H, L, and S in the epithelium at different stages of the cycle by *in situ* hybridization. Fragments of cDNAs coding for cathepsins were prepared by RT-PCR, subcloned into pGEM^R-T vector (Promega), and their authenticity confirmed by direct sequencing. These plasmids were used as the templates to synthesize the sense and anti-sense RNA probes which were labeled by random-primed incorporation of digoxigenin(DIG)-labeled dUTP. Frozen sections of testes were hybridized with DIG-labeled RNA probes. Thereafter, the sections were incubated with a sheep anti-DIG antibody conjugated to alkaline-phosphatase. The antibody bound DIG probe-mRNA duplexes were visualized by an enzymatic reaction. It was found that the sense probes did not yield any positive staining. Using the anti-sense probes, cathepsin L mRNA was found to localize almost exclusively at the basal layer within the cytoplasm of Sertoli cells and spermatogonia in the epithelium. At stages VI-VII of the cycle just prior to the release of elongate spermatids into the lumen, the signal of cathepsin L mRNA was so intense that it formed a complete dark stained precipitate at the basal lamina in the epithelium that encircled the entire tubule. At the beginning of stage VIII just prior to spermiation, no staining of cathepsin L mRNA was seen in the epithelium. For cathepsins D and S, their mRNAs were found abundantly in the cytoplasm of Sertoli cells and spermatocytes in almost all stages but peaked at stages VII and VIII of the cycle. Very few cathepsin H mRNA was found in the epithelium but largely restricted to the interstitium. Conclusion: (i) the expression of multiple cathepsin mRNAs in the testis is stage specific; and (ii) GC express multiple cathepsin mRNAs suggesting they may play a more active role than anticipated in their migration.

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IDENTIFICATION, MOLECULAR CLONING AND CHARACTERIZATION OF SPERM TAIL AUTO-ANTIGEN SA-ODF75. ((M.S.Dubova-Mihailova, K.L.Klotz, M.L.Baran, C.J.Flickinger, J.C.Herr)) Dept. of Cell Biology, University of Virginia Health Sciences Center, Charlottesville, VA 22908

Rat hyper-immune serum raised against rat epididymal spermatozoa was used to identify and clone a highly conserved sperm antigen (SA-ODF75) by screening a human testis cDNA library. The major mRNA transcript for SA-ODF75 encodes a 75 kD protein. High stringency Northern blot analysis of mRNA from 16 human and 22 baboon tissues reveals testis specificity of expression of SA-ODF75. In Western blots of human, mouse and rat sperm extracts rabbit poly-clonal serum raised against human recombinant SA-ODF75 identifies a number of protein bands with Mr 40-43, 53-55, 63-66, 73-75, 85-87 kD. Most of these immunoreactive proteins exist in multiple iso-forms detectable on 2D blots. Comparative immuno-staining of 1D and 2D Western blots of human sperm extracts with rabbit anti-SA-ODF75 anti-serum, rat hyper-immune anti-rat sperm serum or rat post-vasectomy serum prove that SA-ODF75 is one of the main human sperm proteins identified by rat post-vasectomy and hyper-immune sera, suggesting that SA-ODF75 is a major highly conserved sperm auto-antigen. Indirect immuno-fluorescence with affinity purified rabbit anti-SA-ODF75 antibodies localize SA-ODF75 to the middle piece and proximal principal piece of the tail, with no detectable staining of the distal end of the principal piece. Immuno-electron studies localize SA-ODF75 to the cortical area of outer dense fibers and their associated structures. Sequence and predicted secondary structure similarity of SA-ODF75 C-terminal domain to non-muscle myosin heavy chain tail suggests that it may be a member of the myosin family. (Supported by NIH HD29099)

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CENTRIN EXPRESSION AND LOCALIZATION IN THE DEVELOPING MOUSE TESTIS DURING SPERMATOGENESIS. ((J.N. Glantz and J.L. Salisbury)) Tumor Biology Program, Mayo Foundation, Rochester, MN 55905.

The Ca^{2+}-binding protein centrin is a component of centrosomes, mitotic spindle poles, centrioles, and the basal bodies of cells with motile cilia or flagella. Previous studies have shown that centrin is essential for spindle pole separation in budding yeast and for flagellar excision in the green alga Chlamydomonas. During spermatogenesis, specialized microtubule-based structures play essential roles in forming the meiotic spindle, reshaping the nucleus, and forming the sperm flagellar axoneme. We have used RT-PCR, Western blotting and immunohistochemical techniques to elucidate the association of two mouse centrin isoforms, mCen1 and mCen2, with specialized microtubule-based structures of spermatogenic cells. Using oligonucleotide primers specific for either mCen1 or mCen2 cDNAs, we have shown that mCen2 is constitutively expressed in a variety of mouse tissues, while mCen1 expression is limited specifically to developing testicular cells. Semi-quantitative RT-PCR analysis reveals that the expression of mCen1 mRNA increases dramatically during spermatogenesis coincident with the onset of meiosis in male germ cells on post-partum day 14. Immunoprecipitation and Western blot analysis of adult and neonatal testis lysates using anti-centrin antibodies demonstrates differential expression of mCen1 and mCen2 at the protein level. In mature spermatozoa, immunostaining with anti-centrin antibodies labeled both the basal body and proximal centriole. Phosphospecific centrin antibodies label phosphorylated centrin along a larger caudal portion at the basal plate of mature sperm nuclei. These results show that centrin is associated with the microtubule based structures of mature sperm. These studies also suggest that mCen1 plays a unique functional role in testis development through its specific expression in meiotic and post-meiotic sperm cells while mCen2 maintains a more-or-less constant level of expression in somatic cells.

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A QUANTITATIVE STUDY OF THE PROPORTION OF POLYSOMAL mRNA AND RIBOSOME-SPACING OF 16 mRNAs IN MOUSE TESTIS. ((L.M. Cataldo, M.A. Mastrangelo, and K.C. Kleene)) Department of Biology, University of Massachusetts Boston, Boston MA 02125.

The vast majority of mRNAs in meiotic and haploid spermatogenic cells exhibit high levels of translationally inactive free-mRNPs, indicative of a block to the initiation of translation. To investigate the mechanism of this inhibition, seminiferous tubules from adult mice were cultured briefly, and the distributions of 16 mRNAs expressed in various testicular cell-types were analyzed by sedimentation of cytoplasmic extracts on sucrose gradients and measurement of the levels of each mRNA in gradient fractions by densitometry and phosphoimaging of northern and slot blots. The results reveal that the proportions of various mRNAs sedimenting with polysomes range from less than 15% to greater than 80%, demonstrating that mRNA-specific mechanisms regulate the initiation of translation. Most mRNAs in meiotic and early haploid spermatogenic cells and Sertoli cells are translated on polysomes in which ribosomes are spaced about 80-100 bases apart on the coding region, the typical spacing in somatic mammalian cells. However, the ribosome spacing on protamine 1 and 2 mRNAs is much closer, 30-40 bases, demonstrating that in late haploid cells the rate of translational initiation is unusually rapid relative to the rate of elongation. Slowing translational elongation with cycloheximide has negligible effects on the proportions and ribosome-spacing of polysomal mRNAs in meiotic and haploid cells, indicating that competition for initiation factor eIF-4E, a major form of translational control in somatic cells, does not account for the high levels of these mRNAs in free-mRNPs.

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ISOLATION OF THE RAT SPERMATID MANCHETTE: BIOCHEMICAL AND STRUCTURAL CHARACTERIZATION. ((K. Mochida, L.L. Tres, and A.L.Kierszenbaum)) Department of Cell Biology and Anatomical Sciences, CUNY Medical School, New York, NY 10031.

We have developed a procedure for the isolation of intact manchettes from rat spermatids of spermiogenic steps 8-14. The procedure consists in the isolation of seminiferous tubular segments from spermatogenic stages VIII-XIV, followed by fractionation of detergent-solubilized specimens by ultracentrifugation using a 33/50% Percoll gradient in the presence of a buffer that stabilizes the integrity of microtubules and microtubular-associated proteins. Samples resulting from the fractionation procedure were monitored by whole-mount electron microscopy after negative staining, indirect immunofluorescence using tubulin antisera, and two-dimensional PAGE and immunoblotting using antisera to α and β_{1-3} tubulin isotypes, tyrosinated and acetylated α tubulin, β actin, cytoplasmic dynein, *Sak57* (for *spermatogenic cell/sperm-associated keratin*, Mr 57 kDa/pI 5.0-5.9) and *TBP-1* (for *tat-binding protein-1*). We have previously reported that *Sak57* is a soluble acidic keratin associated with microtubules of the manchette (Kierszenbaum et al.; Tres & Kierszenbaum Molec. Reprod. Dev., in press). *TBP-1*, a protein regarded as a transcription activator, also contains an ATPase-like domain (Rivkin et al., submitted for publication). Immunoblotting experiments demonstrate the presence in fractionated manchettes of *tyrosinated* α *tubulin*, β_{1-3} and several microtubule-associated proteins, including *Sak57*, *TBP-1*, *cytoplasmic dynein* and β *actin*. β actin was visualized by immunogold electron microscopy in association with microtubules of the manchette (steps 14-17), flanking the paraaxonemal-aligned mitochondria along the middle piece of the tail. Whole-mount electron microscopy demonstrated microtubules associated with structures regarded as microtubule-associated proteins. Results of this study indicate that this newly developed procedure for the isolation of manchettes should facilitate an understanding of the molecular interaction between tubulin isotypes, cytokeratins and functional proteins during sperm nuclear shaping events.

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ALKALINIZING AGENTS INCREASE THE RESPONSE OF THE HUMAN SPERM ACROSOME TO THE AGONIST, PROGESTERONE. ((Nicholas L. Cross)) Department of Anatomy, Pathology, and Pharmacology, Oklahoma State University, Stillwater OK.

During incubation in vitro, human sperm gradually become able to acrosome-react in response to the agonist, progesterone (P). Sperm must lose unesterified cholesterol to become responsive, but other aspects of the process are not well understood. These experiments tested the possible involvement of intracellular pH (pH_i) in the acquisition of responsiveness. Sperm were incubated 24 hr in vitro in a modified Tyrode's medium containing 26 mg/ml bovine serum albumin in the absence (Con) or presence (Cho) of 1 μM cholesterol (to inhibit sperm cholesterol loss and development of acrosomal responsiveness). Trimethylamine (TMA, 20 mM) + P (1 μg/ml) induced 21 ± 4% (mean ± sem, n = 3) of Cho sperm to acrosome-react, compared to 2 ± 1% with P alone. NH_4Cl and monensin gave similar results. Con sperm responding to P increased from 26 ± 2% to 42 ± 6% in the presence of TMA. In dose-response studies, the size of the incremental population of Cho sperm responding to TMA + P compared to P alone always matched the incremental population of Con sperm, suggesting that TMA was not overcoming inhibition by high cholesterol, but rather permitting response of a special subset of sperm. Freshly ejaculated sperm did not respond to TMA + P. The results suggest that elevated pH_i can increase acrosomal responsiveness of incubated sperm, but it is probably not sufficient to make fresh or cholesterol-inhibited sperm responsive. Further, high sperm cholesterol does not prevent all time-dependent changes related to acrosomal responsiveness. Supported by NIH grant HD30763.