INVESTIGATING OOCYTES AND INTESTINE LINEAGES BY THE RECONSTRUCTION OF CELL LINEAGE TREES

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The embryonic development and post-natal maintenance of the female germline has recently become a subject of active scientific debate, with doubts as to the clonal relation between adult stem cells and primordial germ cells as well as conflicting evidence of post-natal oocyte renewal. Here we analyze acquired somatic mutations to reconstruct lineage trees of hundreds of oocytes as well as of other cell types like the intestine that serves as a validation for the reliability of this method. These cells were sampled from mismatch-repair deficient mice at various ages. In the reconstructed lineage trees we validated the reliability of this method by the examination of different topological aspects of the intestinal reconstructed tree which were validated by other methods that were employed in the past in this tissue. In the oocytes lineage we have shown that oocyte cluster distinctly from cells of bone marrow origin, show no lineage barrier between ovaries and increase in depth (number of cell divisions since the zygote) with mouse age, an increase accelerated after unilateral ovariectomy. The deeper oocytes in older mice may be pre-natal, entailing depth-guided oocyte maturation or post-natal, entailing oocyte renewal in the adult mouse. Our results have important implications to the understanding of the lineage origins of adult stem cells and to oocyte aging.

THE REGULATION AND TOPOLOGY OF UBIQUITINATION IN MAMMALIAN OOCYTES DURING MEIOSIS

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The cyclin-dependent kinase 1 (CDK1) is a master regulator meiosis in oocytes. The accumulation of Cyclin B1, upon reinitiation of meiosis, brings about CDK1 activation. Prior to anaphase I, the ubiquitination and subsequent proteasomal degradation of Cyclin B1, results in CDK1 inactivation, a prerequisite for the extrusion of the first polar body (PBI), representing the completion of the first meiotic division. Proteasomal degradation in mammalian cells is mediated by the formation of polyubiquitin chains, which are known to be linked through lysine 48 of the ubiquitin protein. However, ubiquitin has 6 other lysine residues capable of chain formation, the functions of most of which are unknown. We aimed at investigating the control of ubiquitin-mediated degradation on CDK1 activity during meiosis, in mouse oocytes. In particular, we explored the ubiquitin chain topology needed for PBI emission. We demonstrate herein that while the extrusion of polar body is completely inhibited by a global blockage of proteasomal degradation, the addition of a pharmacological inhibitor of CDK1 can induce both cytokinesis and anaphase. This effect is abrogated upon the introduction of a PLK1 inhibitor. Astonishingly, the extrusion of PBI in the presence of Proteasome and CDK1 inhibitors can be reversed when the CDK1 inhibitor is washed. In addition, by micro-injecting single-lysine ubiquitin mutants, we found that the K11R, but not the K48R mutated ubiquitin, blocked PBI extrusion, indicating that the lysine 11 topology, rather than the classic lysine 48 topology, is involved this process. Furthermore, we show that the K11R-induced inhibition of PBI emission can be rescued using CDK1 inhibitor. Taken together, our data sheds light on the nature of *in vivo* ubiquitination, and its control of CDK1 during mammalian meiosis.

TRANSCRIPTIONAL REGULATION OF MITOCHONDRIAL PROTEASES PROTECTING MITOCHONDRIAL HOMEOSTASIS DURING STEROID HORMONE SYNTHESIS

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Rational: High efficiency vital steroid hormone biosynthesis requires intense expression of StAR (Steroidogenic Acute Regulatory) protein known to facilitate transfer of cholesterol substrate to the inner mitochondrial membranes, where steroidogenesis ensues. StAR activity is terminated by its import into the organelle matrix, where its rapid accumulation can create a cytotoxic 'protein-overload stress'. To prevent that, the mitochondrial quality control machinery degrades StAR by concerted action of nuclear-encoded ATP-dependent protease/chaperone complexes, LON and AFG3L2 (L2). We sought the mechanism by which 'StAR-overload stress' generates mitochondria-to-nucleus communication culminating in upregulation of LON and L2 transcription to better protect the mitochondria during such stress.

Results: ***(a)*** *Up to a 3-fold increase of StAR degrading mito-protease gene products (mRNA/RT-qPCR and Western/protein) were observed in* rat ovarian follicles induced to ovulate *in vivo* (eCG/hCG administrations) when *Lon*, *Afg3l2*, and other mitochondrial proteases (*Spg7*, *Clpp* and *Yme1l1)* were studied; selective upregulation was observed at the level of *Lon* and *Afg3l2* expression in ovarian granulosa cells treated with FSH and testosterone. ***(b)*** Functional mapping of human *LON* promoter revealed proximal regions necessary for activity and consensus binding sites (EMSA) for NRF-2 necessary for its activity. In addition, we have identified an upstream strong inhibitory element assessed to bind a key repressor factor E2F-4.

Discussion: Using powerful endocrine cell models, our findings suggest a regulated mechanism that can modify the expression of normally regarded house-keeping genes encoding mitochondrial proteases. To do so, the mitochondria somehow generate as yet to be defined signal that activates in the nucleus at least two pivotal of transcription factors, i.e., NRF-2 that is required for diverse mitochondrial functions such as respiration, biogenesis, mtDNA transcription and replication, and E2F-4 known to be a critical repressor in G_1-arrested cells. Thus, hormone producing cells of the adrenal and the gonads provide a unique model to study transcriptional regulation of mitochondrial proteases engaged in maintenance of the organelle homeostasis under crisis conditions such as heat-shock, hypoxia, aging (LON), and neuronal disorders (SPG7, AFG3L2).

MENOPAUSAL TRANSDERMAL ESTROGEN AND INTRAUTERINE LEVONORGESTREL DOES NOT INCREASE CRP LEVELS WHEREAS ORAL HORMONE TREATMENT MAY

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Background: The controversy regarding the pros and cons of menopausal hormone treatment [HT] is still ongoing since the intriguing results of the WHI study.Laboratory and experimental evidence have shown that infl ammatory processes play a central role in the development, progression and outcomes of atherosclerosis. Several studies suggest that patients at high risk of developing atherothrombotic disease suffer from chronic systemic inflammation. More specifically, increased levels of C-Reactive Protein (CRP), a marker of systemic infl ammation, have been associated with a higher risk of cardiovascular morbidity and mortality. CRP levels are associated not only with the presence of atherosclerosis but also with its clinical severity.

Objective: To assess CRP levels in menopausal patients treated with oral HT vs menopausal transdermal estrogen [TDE] and intrauterine levonorgestrel [Mirena IUD].

Methods: Menopausal women [n=148] under various forms of HT, or controls, have undergone CRP measurement.

Results: Patients receiving oral conjugated equine estrogen + gestagens [n=25] had mean± SD CRP level of 5.36±2.9, significantly higher than the CRP levels in all the other treatment groups or control [**P<0.001**]. The combination of TDE and Mirena had normal mean CRP concentration,not significantly different from control, or TDE and progesterone[Evorel], oral estrogen + drospirenon [Angelique], or TDE and vaginal progesterone.

Conclusion: The significantly increased CRP levels associated with oral conjugated equine estrogen + gestagens menopausal treatment may possibly represent an increased risk of CV morbidity and mortality as suggested by the WHI study. Other forms of HT, especially the TDE and intrauterine or vaginal gestagens are not associated with high CRP levels.

THE SPECTRUM OF GONADAL AND HYPOTHALAMIC FUNCTION IN ADOLESCENTS AND ADULTS WITH PRADER-WILLI SYNDROME (PWS)

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Background: Hypogonadism, a cardinal feature of Prader-Willi syndrome (PWS) is characterized by variable clinical manifestations. The etiology is heterogeneous and there is no consensus regarding treatment.

Objective: To characterize the spectrum and causes of hypogonadism in a cohort of PWS adolescents and adults.

Methods: We measured reproductive hormonal profiles of 19 males (m) and 16 females (f) ages 15 to 32 years with genetically confirmed PWS. Puberty was assessed by Tanner staging; blood was sampled for gonadotropins, sex-steroids and the gonadal-specific peptides, inhibin B (INB) and anti-Mullerian hormone (AMH).

Results: We found four distinct hormonal profiles based on INB and FSH levels: Group A (m:f; 8:1): hypergonadotrophic (primary gonadal) hypogonadism with elevated FSH levels (> 15 IU/l) and undetectable inhibin B. Group B (m:f; 4:4): hypogonadotrophic hypogonadism with FSH < 0.5 IU/l and inhibin B < 7 pg/ml. Group C (m:f; 3:5): partial gonadal and hypothalamic function with inhibin B > 20 pg/ml and FSH 2-10 IU/l. Group D (m:f; 4:6): mild hypothalamic and severe gonadal dysfunction (FSH 0.5-10 IU/L and INB < 20 pg/ml). There were significantly more males in group A vs C or D ($P<0.05$). Mean breast Tanner stage and testosterone levels were highest in group C ($p<0.03$), mean LH was highest in group A ($p<0.001$), and mean AMH was highest in group B ($p<0.005$). No differences were found in genetic subtype, age and BMI among the four groups.

Conclusion: We characterized four distinct phenotypes of hypogonadism in PWS adolescents and adults ranging from primary gonadal to hypothalamic hypogonadism; the minority had gonadotropin deficiency. Determining individual reproductive hormone patterns, including INB, may be important for assessing fertility feasibility in women and for recommending contraception to females or hormonal replacement therapy for both genders.

THE ROLE OF SPERM LIGANDS IN FORWARD AND HYPERACTIVATED MOTILITY, CAPACITATION AND ACROSOME REACTION, IN HUMAN SPERM

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Introduction: Mammalian sperm are activated by sperm ligands but the nature of the stimulating ligands is still unknown. MAPKs are key regulatory enzymes in signal transduction. We have recently characterized human sperm MAPKs, and implicated ERK in forward and hyperactivated motility and acrosome reaction (AR). Here we examine the effect of EGF and TGF-β3 upon ERK activation and the role of ERK in forward and hyperactivated motility, capacitation and acrosome reaction.

Patient/Methods: Sperm samples from healthy donors and patients were obtained from Sheba Medical Centre Sperm Bank, Tel-Hashomer Hospital.

Results: EGF and TGF-β3 activated ERK within 5 minutes. The effect was persistent and still detectable in sperm after capacitation. Incubation of normal spermatozoa with EGF, increased forward motility within the first 5 minutes, and hyperactivation after capacitation. Incubation with TGF-β3 increased both forward and hyperactivated motility within the first 5 min. Later, we examined whether the ligand-induced motility is mediated *via* ERK-dependent mechanism, by adding, U0126 a selective inhibitor of MEK. Indeed, pre-incubation with the inhibitor reduced the percentage of motile sperm and abolished the effect of the ligand on forward motility. Both EGF and TGF-β3 stimulated also sperm AR and the effect was mediated by ERK.
We have also identified several proteins that were phosphorylated on tyrosine during capacitation and the effect was markedly reduced in the presence of a MEK inhibitor.

Conclusions: EGF and TGF-β3 stimulate sperm motility and AR *via* an ERK-mediated cascade. Protein tyrosine phosphorylation, a hallmark of capatitation is also mediated by ERK.

LONG ACTING HORMONES DESIGNED BY GENE FUSION AND GENE TRANSFER ARE SAFE FOR USE IN CLINICS

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Glycoprotein hormones are used clinically in the treatment of many diseases. One major issue regarding the clinical use of many peptides is their short half-life span in the body, due to the rapid clearance from the circulation. The low stability of peptides has thus often posed a difficulty to researchers and hindered their adoption in potential medical applications. Thus, at the clinical level, there is a need for a regime of frequent injections of the peptides into the patients to overcome this low stability factor. The major strategies for overcoming this problem by pharmaceutical companies are based on chemical techniques and using specific peptidase inhibitors or cocktails. To overcome this problem, we used genetic engineering techniques that have been found successful for designing long acting hormones. Using overlapping PCR and gene transfer techniques, we succeeded to add the signal sequence of *O*-linked oligosaccharides to the coding sequence of the hormones. The cassette gene that have been used contains the sequence of the carboxyl-terminal peptide (CTP) of human chorionic gonadotropin β (hCGβ) subunit. The CTP contains 28 amino acids with four *O*-linked oligosaccgaride recognition sites. It was postulated that the *O*-linked oligosaccharides add flexibility, hydrophilicity and stability to the protein. On the other hand it was suggested that the four O-linked oligosaccharides play an important role in preventing plasma clearance and thus increasing the half-life of the protein in the circulation. Using this strategy we succeeded to ligate the CTP to the coding sequence of follitropin (FSH), thyrotropin (TSH), erythropoietin (EPO) growth hormone (GH) and thus to increase the longevity and bioactivity of these proteins in-vivo. Interestingly, the new analog of FSH was found not immunogenic in humans and it is already passed successfully clinical trials phase III. Moreover, FSH long acting was approved by the European Commission (EC) for treatment of fertility. All designed variants were successfully expressed in Chinese Hamster Ovary Cells (CHO). Designing long acting peptides will diminish the cost of these drugs and perhaps reduce the number of injections in the clinical protocols.