Alternative splicing of Lysyl Oxidase-like 4 in ovarian carcinoma

Shulamit Baror-Sebban [1], Regina Golan-Gerstl [2] , Rotem Karni [2], Ben Davidson [3], Reuven Reich [1]

[1] *Institute of Drug Research, Faculty of Medicine, Hebrew University, Jerusalem*
[2] *Department of Biochemistry and Molecular Biology, Faculty of Medicine, Hebrew University, Jerusale*
[3] *Department of Pathology, National Hospital–Norwegian Radium Hospital, of Oslo, Montebello*

Introduction: Lysyl oxidase (LOX) is an amine oxidase that is usually synthesized and secreted by fibrogenic cells. Four LOX-like (LOXL) genes have been identified so far in mammalian genomes, encoding four different LOX-like proteins: LOXL1, LOXL2, LOXL3 and LOXL4. All members of the LOX family show a highly conserved C-terminus region that contains the catalytic domain. The N-terminus of the LOX isoforms is less conserved among the different members and is thought to determine the individual role and tissue distribution of each isoenzyme. LOXL4, the least studied member of the LOX-like family enzymes, undergoes a process of alternative splicing in cancer, in a site- and stage-specific manner that we have previously shown. The purpose of the current study was to uncover the splicing mechanism that is responsible for this process.

Patients/ Methods: I. ShRNAs for four splicing factors: SF2/ASF, SRp55, hnRNP-A1 and hnRNP-A2, were transfected in two cell lines: U-87 MG cell line (human glioblastoma) and NCI-H460 (human large-cell lung carcinoma). II. Over-expression of SF2 was performed in MST0-211H cell line (human malignant mesothelioma), HeLa cell line (human epithelial cervical cancer) and MCF10A cell line (human mammary epithelial line). III. Western blotting for SF2/ASF and tubulin. IV. RT-PCR for LOXL4 full length, splice-variant1 (splv1) and splice-variant2 (splv2) mRNA expression.

Results: We examined LOXL4 expression in U-87 MG cells. When untreated, these cells express the full length and splv2, almost equally. The silencing of two factors, SF2/ASF and hnRNP-A1, resulted in a dramatic change in the expression pattern of LOXL4. For both silenced factors, LOXL4 full-length mRNA expression was much stronger, while the shortest variant, splv2, completely vanished. The silencing of hnRNP-A2 led to a smaller decrease in splv2, while SRp55 silencing did not seem to change LOXL4 splicing. In NCI-H460 cells, which normally express small amounts of all variants, no significant changes were found following silencing. In an attempt to further establish the splicing factor responsible for LOXL4 splicing, we over-expressed SF2/ASF in MST0-211H cells, which normally express only the full length LOXL4. Expression of SF2/ASF resulted in the appearance of splv2, while dramatically reducing the expression of the full length. Similar results were seen in HeLa cells. Over-expression of SF2/ASF in MCF10A cells, which untreated, have the unique quality of expressing splv2 alone, caused only a slight increase in the expression of splv2.

Conclusions: These results demonstrate for the first time, that LOXL4 is a direct target of the splicing factor SF2 SF2/ASF. Furthermore, in concordance with our previous in-vivo findings, it can be concluded that LOXL4 splicing occurs similarly in other epithelial cancer types, such as breast cancer and mesothelioma.

Preclinical analysis of IGF-IR antibody MK-0646 in endometrial cancer

Conny Bitelman [1], Rive Sarfstein [1], Zohar Attias [1], Ami Fishman [2], Ilan Bruchim [2], Haim Werner [1]

[1] *Department of Human Molecular Genetics and Biochemistry, Sackler School of*
[2] *Gynecologic Oncology Unit, Meir*

Introduction: Endometrial cancer is one of the most frequent gynecological cancers in Western countries. The involvement of the insulin-like growth factors (IGFs) in the initiation and progression of endometrial cancer has been well established. The IGF-I receptor (IGF-IR), which mediates the proliferative and antiapoptotic activities of IGF-I and IGF-II, emerged in recent years as a promising molecular target in cancer therapy. The aim of the present study was to evaluate the hypothesis that interfering with the IGF-IR signaling pathway in endometrial cancer could revert the transformed phenotype, decrease proliferation, induce apoptosis, and render the cells more sensitive to chemotherapy. To this end we used a recently developed humanized monoclonal antibody (MK-0646, Merck Oncology), directed against IGF-IR. MK-0646 was shown to block IGF-I binding to the receptor, leading to IGF-IR degradation.

Patients/ Methods: To evaluate the effect of IGF-IR inhibition on IGF-I-mediated signaling, human endometroid (ECC-1) and serous papillary (USPC-1) endometrial cancer cell lines were treated for various periods of time (40 min, 1 h, 3 h, 5 h) with antibody MK- 0646, in the presence of IGF-I during the last 10 min of the incubation period.

Results: Results of Western blots using antibodies against total and phospho-IGF-IR, AKT, and ERK showed that MK-0646 decreased the IGF-I-stimulated phosphorylation of IGF-IR, AKT and ERK in both ECC-1 and USPC-1 cells. In addition, MK-0646 induced a significant decrease in total IGF-IR levels. To evaluate the potential effect of IGF-IR inhibition on apoptosis, cells were treated with IGF-I for 24-48 h, in the absence or presence of MK-0646, after which apoptosis was assessed by Caspase-3 and cleaved PARP measurements. Results obtained showed that MK-0646 abrogated the antiapoptotic activity of IGF-I

Conclusions: Taken together, our results suggests that specific IGF-IR blockade could be a useful therapeutic approach in endometrial cancer.

Transcriptional regulation of gonadotropin genes by GnRH through its effects on histone H3 methylation and phosphorylation

Andrea Wijeweera [1], Philippa Melamed [1]

[1] *Faculty of Biology, Technion*

Introduction: Luteinizing hormone and follicle stimulating hormone control reproductive development and function. Both hormones comprise a common α- and a hormone specific β-subunit, which are regulated by the gonadotropin releasing hormone (GnRH). We have demonstrated that GnRH can regulate gonadotropin subunit gene transcription at the level of chromatin, through the displacement of histone deacetylases. This allows subsequent histone acetylation. We hypothesize that transcriptional activation of the subunit genes by GnRH involves the induction of a sequence of histone modifications, including monoubiquitination of histone H2B at lysine K120 (H2BK120ub), trimethylation of histone H3 at lysine 4 (H3K4me3) and lysine 36 (H3K36me3) and/or phosphorylation of H3 at serine 10 (H3S10p), modifications previously shown to be implicated in yeast and mammalian transcriptional regulation.

Results: Our data shows that GnRH increases nuclear protein levels of H3S10p and phosphorylated MSK1 in gonadotrope cells. As MSK1, which is activated by ERK or p38MAPK, targets H3S10, this suggests that GnRH may activate subunit gene transcription through regulating H3S10p. H3S10p is reported to be required for histone acetylation by GCN5, which we have found is associated with the LHβ promoter. ChIP studies revealed the presence of H3K4me3 on all three subunit gene promoters. While nuclear protein levels of H3K4me3 are unaltered by GnRH, ChIP studies normalized against levels of total H3 present at the promoters, demonstrate an increase in H3K4me3 at the subunit gene promoters after GnRH treatment. GnRH also increases nuclear protein levels of H3K36me3 and H2BK120ub, modifications implicated in transcription elongation, suggesting that GnRH may regulate elongation through controlling these modifications.

Conclusions: These results therefore suggest that GnRH regulates gonadotropin subunit gene transcription through the induction of various histone modifications. The function of, and mechanism behind, each of these modifications are currently being studied

Sustained activity of the EGF receptor is an absolute requisite for LH-Induced oocyte maturation and cumulus expansion

Judith Elbaz [1], Yitzhak Reizel [1] , Nava Dekel [1]

[1] *Department of Biological Regulation, Weizmann Institute of Science, Rehovot, Israel*

Introduction: Maturation of the oocyte and expansion of its surrounding cumulus cells constitute part of the responses of the ovarian follicle to the preovulatory surge of LH. It was previously shown that activation/phosphorylation of the epidermal growth factor receptor (EGFR) mediates the ovulatory response to LH. Unlike other systems in which the EGFR is rapidly dephosphorylated, in the ovary, it stays phosphorylated for few hours following LH stimulation. We hypothesized that this sustained activity of the EGFR is required to mediate LH action in the ovary through the continuous activation of its downstream ERK1/2.

Patients/ Methods: Intact preovulatory follicles were isolated from 25-day-old PMSG-primed Wistar female rats. They were exposed to either LH or forskolin in the presence or absence of specific inhibitors of their downstream effectors. At the end of incubation, the follicles were incised, and the cumulus oocyte complexes were monitored for the meiotic status of the oocyte as well as for the extent of cumulus expansion. Activation of ERK1/2, as indicated by its state of phosphorylation, was examined by Western Blot analysis. The expression of prostaglandin-endoperoxide synthase 2 (Ptgs2, also known as Cox2), as well as that of MAPK phosphatases (MKPs), was determined by RT-PCR.

Results: We demonstrated that a short-term exposure of ovarian follicles to LH, as well as a transient activation of the adenylyl cyclase by forskolin, is sufficient to trigger oocyte maturation and cumulus expansion. By contrast, termination of the activity of the EGFR, at time points that are earlier than 3 h of exposure to LH, severely impaired these responses. In addition, the sustained activity of the EGFR was essential for an extended phosphorylation of the ERK1/2 downstream signaling molecules, which by itself is required for oocyte maturation and cumulus expansion. Interestingly, the continuous activity of the EGFR was also necessary to maintain the up-regulation of Ptgs2, an indispensable gene for cumulus expansion. In search for a mechanism that may be responsible for the prolonged ERK1/2 activity in the ovary, we screened the inducible MKPs, which specifically dephosphorylate the active ERK1/2. This experiment revealed, for the first time, that MKP-3 up-regulation is temporally correlated with ERK1/2 dephosphorylation, pointing toward MKP-3 as the potential enzyme responsible for ERK1/2 shutdown in this system.

Conclusions: Our data demonstrate that a prolonged activity of the EGFR is absolutely required to mediate the LH-induced responses in rat preovulatory follicles. We propose that this mechanism allows translation of the short systemic surge of LH into deferred, multiple responses that bring about the spatiotemporal coordination of a wide range of events, collectively defined as ovulation. This study highlights the fact that the sustained, nonclassical activity of the EGFR-ERK1/2 pathway is necessary
for the gonadotropins-induced ovulatory response.

The Indispensible role of ROS in Ovulation

Ketty Shkolnik [1], Ari Tadmor [1] , Nava Nevo [1], Dalia Galiani [1], Shifra Ben-Dor [2], Nava Dekel [1]

[1] *Department of Biological Regulation, Weizmann Institute of Science, Rehovot 76100, Israel*
[2] *Department of Biological Services, Weizmann Institute of Science, Rehovot 76100, Israel*

Introduction: Ovulation, that is an essential prelude for successful reproduction is stimulated by the preovulatory surge of the pituitary luteinizing hormone (LH). The biochemical and molecular effects of this gonadotropin in the preovulatory follicle culminate in the release of a mature ovum surrounded by the cumulus cells. Since ovulation has biophysical and biochemical features that are characteristic of an inflammatory reaction, reactive oxygen species (ROS) may play a role during this process. ROS in the ovary may originate from inflammatory cells, such as macrophages and neutrophils, which are present during ovulation and produce large amounts of free radicals. Ovarian ROS may also be generated by the LH-induced cyclooxegenase (Ptgs2) expression, an enzyme that catalyzes the initial oxidation step in the conversion of arachidonate to prostanoids associated with inflammatory conditions. ROS are also byproducts of monooxegenase reactions mediated by P450 systems in steroidogenic cells. The NADPH dependent generation of superoxide was shown to increase during the early preovulatory phase in the ovary of cycling female mice. Taking this information into consideration, we hypothesized that ROS may be involved in the signaling cascades leading to ovulation.

Patients/ Methods: The role of ROS in ovulation was examined in-vivo in PMSG/hCG-primed mice injected into their ovarian bursa by broad-range anti-oxidants, such as NAC and BHA. An ex-vivo approach of intact ovarian follicles culture was employed to further elucidate the molecular mechanism of ROS involvement in the LH-induced preovulatory responses such as cumulus expansion and progesterone secretion. Western blot analysis was employed to analyze the state of EGFR and MAPK42/44 phosphorylation/activation. Real-time PCR analysis was performed to assess the expression profiles of ovulation essential genes. For this purpose large antral follicles obtained from PMSG-primed mice were incubated with LH in the presence or the absence of antioxidants.

Results: Administration of broad range scavengers of oxidative species such as BHA and NAC into the ovarian bursa of PMSG/hCG treated mice significantly reduced the rate of ovulation. This observation gained further support by ex-vivo experiments performed on isolated intact ovarian follicles. In this system, LH-induced cumulus mucification/expansion, that is a necessary prerequisite for ovulation was prevented by the antioxidants mentioned above. Along this line, H2O2 fully mimicked the effect of LH, bringing about an extensive mucification/expansion of the follicle-enclosed cumulus-oocyte complexes. Progesterone production that is another parameter tightly associated with ovulation was also impaired in isolated follicles incubated with LH in the presence of the antioxidant agents. The inhibitory effect of scavengers of ROS on LH-induced ovulatory responses in the ovarian follicles was also manifested at the molecular level. The LH-stimulated up-regulation of genes such as Ptgs2, Has2, TNFAIP6, Pgr and Cebpb, the expression of which is a prerequisite of normal ovulation, was substantially attenuated upon the addition of the antioxidants to the culture. These antioxidants also inhibited the LH-induced phosphoryilation and activation of the EGFR as well as that of its downstream effector, p42/44 MAPK.

Conclusions: In the present work, we employed in-vivo studies in combination with ex-vivo and molecular analyses to demonstrate the indispensible role of ROS in ovulation as well as in other ovulatory responses such as cumulus expansion, progesterone secretion and specific genes up-regulation and activation. These observations raise the novel idea that reactive oxidants present in the preovulatory ovarian follicles are essential for the ovulatory response.

Designing long acting analogs of glycoprotein hormones using site-directed mutagenesis and gene transfer

Fuad Fares [1], Avri Havron [2] , Eyal Fima [3]

[1] *University of Haifa, ModigeneTech and Carmel Medical Center*
[2] *ModigeneTech*
[3] *ModigeneTech*

Introduction: 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.

Patients/ Methods: To overcome this problem, we used genetic engineering techniques that have been found successful for designing long acting hormones. Using site-directed mutagenesis and overlapping PCR techniques, we succeeded to add the signal sequence of O-linked oligosaccharides to the coding sequence of the hormones. The cassette gene that has been used contains the sequence of the carboxyl-terminal peptide (CTP) of human chorionic gonadotropin □ (hCG□) subunit. The CTP contains 28 amino acids with several proline and serine residues and 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 circulation.

Results: 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. GH-CTP was found to be safe in monkeys and it passed successfully clinical trials phase I in humans.

Conclusions: Ligation of CTO to the coding sequence of the hormone has no effect on expression, secretion and in vitro bioactivity of the hormone. On the other hand, it increases the half-life and bioactivity in vivo. Furthermore, it seems that the chimerical proteins contain the CTP are not immunogenic. Designing long acting peptides will diminish the cost of these drugs and perhaps reduce the number of injections in the clinical protocols.

A novel mutation in GPR54/Kiss-1R leads to GnRH resistants hypogonadotrophic hypogonadism in a highly consanguineous family

Galia Gat-Yablonski [1,2], Revital Nimri [1] , Yael Lebenthal [1], Meytal Bar [1,2], Nicolas de Roux [4], Moshe Phillip [1,2,3]

[1] *The Jesse Z and Sara Lea Shafer Institute for Endocrinology and Diabetes, National Center for Childhood Diabetes, Schneider Children's Medical Center of Israel*
[2] *Felsentein Medical Research Center, Petah Tiqva*
[3] *Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv,*
[4] *Institut National de la Sante´ et de la Recherche Medicale U690, Hoˆpital Robert Debre´ (A.I., C.A.*

Introduction: GPR54, the kiss1 receptor is essential for normal gonadotropin-released hormone physiology and for puberty, and defects in it cause isolate hypogonadotropic hypogonadism (IHH) or precocious puberty.

Patients/ Methods: To identify the genetic cause of IHH in a highly consanguineous family of Israeli-Arab origin

Results: All patients that were available for endocrine analysis showed IHH that was resistant to GnRH stimulation and were treated with partial success. A novel homozygous p.F272S mutation in GPR54 was identified in the affected patients, the unaffected parents or siblings carried the mutation in a heterozygote state. This mutation resides in the 6th transmembrane domain of the receptor in a highly conserved amino acid and was suspected to lead to inactivation of the receptor. Functional analysis measuring activation of inositol specific phospholipase C (PLC) confirmed complete inactivity of the receptor.

Conclusions: These results enlarge the clinical spectrum associated with mutation in the GPR54