Optimizing the efficacy of TRAIL-induced apoptotic cell death in granulosa tumor cell lines

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Human ovarian cancers of granulosa cell origin currently represent approximately 3 to 5% of diagnosed cancers involving the ovary. Nevertheless, there is little known regarding either the etiology or selective treatment of granulosa cell tumors (GCT) as compared to those originating from surface epithelial cells. The ability of TRAIL to target cancer cells for apoptosis without affecting normal cells has generated interest for its potential use in cancer therapy. However, its efficacy in the treatment of ovarian GCTs has yet to be evaluated. Recent *in vitro* studies have demonstrated that treatment with the naturally occurring cytokine, TRAIL, does not induce apoptosis in healthy ovarian granulosa cells, despite the expression of a functional TRAIL death receptor, DR5. Results obtained using two human GCT lines, COV434 and KGN (both of which were established to be p53 wild-type), demonstrate a slight, but significant, increase in TRAIL (100 ng/ml)-induced apoptosis (KGN, fold *vs* control = .90 +/- .02; COV434, fold *vs* control = .89 +/- .015; $p < .05$). This effect was markedly enhanced when granulosa cells were first treated with the conventional chemotherapeutic, cisplatin (25 uM; $p<.001$). Similarly, treatment with paclitaxel (10 nM) also significantly increased the death-inducing ability of TRAIL. Of notable interest was the finding that while treatment with paclitaxel effectively reduced cell viability in a human ovarian surface epithelial cell line (PA-1) following 48 h treatment, paclitaxel treatment alone was ineffective in mediating cell death in both GCT lines (10-100 nM). However, the combinatorial treatment with paclitaxel plus TRAIL led to a significant decrease in cell viability ($p<.001$). These results provide evidence that a greater efficacy in treating GCTs can be achieved with a regime of cisplatin or paclitaxel followed by treatment with TRAIL.

Leishmania infection inhibits chemically-induced macrophage apoptosis

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Leishmania, a parasite that is transmitted by the bite of the sand fly, resides in host macrophages and can cause a variety of disease manifestations. *Leishmania*sis is endemic in 88 countries and can have devastating effects because of its ability to evade its host's immune response. Previous experiments have shown that infection with *L. major* and *L. donovani* inhibits apoptosis in RAW 264.7 cells, a murine macrophage line. Bone marrow macrophages from C57BL/6 mice were infected in place of the RAW 264.7 cells to examine cell viability and apoptotic inhibition in response to *Leishmania* infection in true cells. As well, it has been shown that *Leishmania* are able to inhibit cycloheximide-induced macrophage apoptosis in a strain specific manner. When treated with cycloheximide, an apoptosis inducer, some strains of *L. major* and *L. donovani*

were able to prevent apoptosis entirely, while other strains inhibited the process to a much lesser degree. Actinomycin D and Camptothecin, other known apoptosis inducers, were administered to multiple strains of *L. major* and *L. donovani* infected RAW 264.7 cells. Cells were stained with APO-BrDU and assessed for DNA fragmentation, an indication that apoptosis has occurred, by Flow cytometry. This study found that infected cells of each strain and species were able to inhibit apoptosis almost entirely when treated with both chemicals. Finally, past experiments have shown a decrease in Cytochrome C, a protein released from the mitochondria, in the supernatant of infected compared to uninfected cells. A whole-cell lysis and Western were performed on uninfected and infected cells in media, ethanol, and cycloheximide to probe for Bad and pBad, proteins attached to the mitochondrial membrane, as possible mechanisms for the strain-specific inhibition of apoptosis. All *Leishmania* species and strains were found to significantly down regulate pBad and Bad proteins, suggesting that pBad and/or Bad are not responsible for the species- and strain-dependent differences in inhibition of CHX-mediated apoptosis.

The identification of neural areas involved in anuran social behaviors by quantitative mapping of mitchondrial activity

Anna Creighton

This project focuses on how social exposure affects the auditory system of the anuran brain using the gray treefrog, *Hyla versicolor*. The goal is to identify the midbrain and forebrain auditory areas involved in anuran social behavior. Frogs obtained from a natural environment during the mating season were divided into two groups and either exposed to a chorus recording for four hours at night or presented with continuous white noise. After three weeks, frogs were sacrificed and brains were removed for enzyme analyses. Regional metabolic activity of mitochondrial enzymes will be analyzed using histochemical techniques. Neuron number and size will also be measured. Call exposure may create differences in the metabolic activity of the midbrain and forebrain regions involved in social behavior. This information will be applied to the evolutionary development of social behaviors.

A preliminary ultraviolet dosing regimen does not rescue the hypocalcemic phenotype in 1-alpha hydroxylase knockout mice.

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Vitamin D is required for regulation of serum calcium levels, as insufficient vitamin D status leads to hypocalcemia, resulting in rickets, osteomalacia, and other growth abnormalities. Vitamin D is a steroid hormone acquired either through diet or skin photosynthesis after irradiation with ultraviolet light. Both forms are metabolized through a series of hydroxylation steps to the active form, which binds to the vitamin D receptor

and regulates transcription of calcium-regulatory genes. Previous research has indicated that dietary vitamin D supplementation can rescue the hypocalcemic phenotype of mice with targeted ablation of 1-alpha hydroxylase, which catalyzes the final activation step. However, since no research has studied the effect of UV-B light on the vitamin D status in these mice, our study was initiated to determine the frequency and intensity of UV light exposure to optimally enhance vitamin D status in these mice. Mice were exposed to a variety of doses from weaning (3-5 wks) until adulthood (11-12.5 weeks of age). Serum levels of 25-hydroxyvitamin D and calcium were measured using enzyme immunoassay and colorimetry, respectively, and femurs were analyzed for mineralization and strength using micro-computed tomography and a three-point bending test. Skin sections were histologically assessed for potential negative effects of chronic UV exposure. Results indicate that the initial UV doses tested did not sufficiently increase 25-hydroxyvitamin D or calcium levels, and weakened bone persisted due to decreased mineralization. More trials are needed to determine the UV dose resulting in maximum photosynthesis of vitamin D in order to test whether vitamin D synthesized endogenously can rescue the 1-alpha hydroxylase deficiency phenotype.

Testing for molecular evidence of natural selection on the yellow gene within the Drosophila cardini subgroup

Megan Ericson

For much of the history of the study of evolution, emphasis has been placed mostly on gross morphological features. Recently, however, there has been a shift towards understanding molecular evolution as seen through genetic studies. The understanding of evolution is imperative for conservation efforts. In order to protect the diversity of life that exists on the planet, the first step is to understand how it was developed. One interesting example of evolutionary forces can be found in the Drosophila dunni subgroup of the Caribbean islands in which an interspecific cline in pigmentation can be found. Such clines indicate that natural selection on this trait may have played a role during speciation. A similar cline is found in the Drosophila cardini subgroup, the sister subgroup of the Drosophila dunni, which is found on the mainland of South America as can be seen in the figure. In this subgroup, however, variation in abdominal pigmentation occurs within species. Within this specific project, the yellow gene within species of the Drosophila cardini subgroup was examined for molecular evidence of the forces of natural selection. The yellow gene was chosen due to its role in the pigmentation pathway of Drosophila and because it has been shown to be under selective pressures within the Drosophila dunni subgroup. To ensure that a fair representation of genetic variation in yellow was shown, several individuals were analyzed from each population and phenotype within that species to ensure adequate sampling. A portion of the yellow gene was amplified and purified for sequencing. These sequences were compared and then analyzed for the molecular signature of natural selection. Preliminary results indicate that yellow is not under selective pressure within this subgroup. This conclusion is interesting as it demonstrates that interspecific divergence of Drosophila pigmentation patterns among closely related species groups is

mediated by diverse molecular mechanisms. Future studies examining genetic variation in other candidate pigmentation loci, such as omb, are underway.

Exploration and development of vitamin-D-receptor-related breast cancer research methods

Jeremy Fagan

The role of the Vitamin D Receptor (VDR) protein and 1-α,25-dihydroxycholecalciferol [1,25 (OH)2-D3]is one that is of significant importance in the study of mammary tissue tumorigenesis. Previous research has examined the preventative and therapeutic effects of VDR and 1,25 (OH)2-D3 against breast cancer. Current VDR research models include transgenic VDR knockout mice, Human Mammary Epithelial (HME) cell lines and human breast cancer cell lines. In previous studies, VDR Knockout mice have proven to be problematic due to their need to be on a special calcium diet and adverse skin and hair deformities that interfere with the study of the role of VDR in mammary tissue. A transgenic mouse model that localizes the knockout of the VDR gene to the mammary tissue would eliminate these problems. This was achieved through the MMTV-Cre/LoxP Recombination System and a qPCR assay from the mammary, hepatic and renal tissue that showed the tissue specific knockout was localized to the mammary gland with a functional VDR still found in the renal and hepatic tissue. The effects of 1,25 D3 and an apoptotic inducing agent, Etoposide, on normal human mammary epithelial cells were performed to determine the possibility of crosstalk between VDR and p53. Western Blotting and qPCR determined the effects on gene expression and protein levels of p53, p21 and VDR at various time points. T47D breast cancer cells were stably transfected with a vector that caused the knockdown of the expression of 1α-hydroxylase, the enzyme responsible for attaching the 2nd hydroxyl group to Vitamin D3 converting it to its active form that binds to VDR. A total of five stable transfected T47D cell lines were generated. Future studies will investigate the effects of knocking down 1α-hydroxylase in these breast cancer cells.

Prevention of transgene silencing and photoconversion of Kaede in Danio rerio

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The zebrafish is a popular model organism because it is amenable to transgenic analysis. Transgenes are a powerful approach to label either a specific subset of cells or a broad pattern in an animal. The Hyde lab recently demonstrated that at least two different ubiquitous promoters were silenced in the adult. Therefore, developing an approach to consistently and effectively prevent transgene silencing is a critical need in developing transgenic animals. One possible solution is to flank the transgene with insulator sequences to prevent chromatin silencing from moving into the transgene. To determine if SCS/SCS' insulators can prevent the silencing of ubiquitously expressed

transgenes in the adult transgenic zebrafish, I generated expression constructs that contain these insulators flanking transgenes. The reporter transgenes used were *XIEe1a1:EGFP*, *CMV:CreRG*, *CMV:FLPeGR*, and *CMV:FLPeRG*. The reporter elements were flanked by either SCS/SCS' sequences in both possible orientations. These constructs will be used to generate transgenic zebrafish lines. Since we know that these promoters are silenced in the central nervous system, if subsequent generations of the transgenic zebrafish show expression in the central nervous system instead of being silenced, then we would have demonstrated that the SCS/SCS' insulators prevent transgene silencing. Insulators will then allow researchers to consistently label cells through the use of transgenes. The next step will be to identify a reporter to express from the ubiquitous promoter for cell lineage analysis. I am currently testing the feasibility of the Kaede protein for this purpose. Kaede fluoresces green but is stably converted by UV light to a red fluorescing protein. I am using transgenic zebrafish lines that express Kaede to determine the efficiency of the photoconversion and the persistence of the red fluorescence in the retinal neurons. A combination of these, and other techniques developed in the Hyde lab, will allow us to label the endogenous retinal stem cells and follow the cellular and molecular pathways as they regenerate damaged retinas.

Localization of NPY Y1 Receptors in Xenopus laevis Olfactory Tissue

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The feeding behavior of vertebrate organisms is largely influenced by olfactory activity. Olfaction is modulated by a class of neuromodulators called neuropeptides. A previous study carried out by Mousley and others implicated neuropeptide Y (NPY) as a mediator in odorant responses in the olfactory epithelium of hungry animals. The mechanism by which NPY mediates appetite control in response to olfactory signals, however, is not thoroughly understood. This study begins the search for a probable mechanism assuming NPY has its mediating effects by binding to NPY cell receptors. Previous study of the nose and brain tissues of hungry axolotls using Southern gel analysis revealed distinct Y1 receptor mRNA bands. This initial data suggests the Y1 mRNA to be expressed in olfactory cells of *Xenopus laevis* as well. Thus, the olfactory tissue of hungry versus fed *X. laevis* will be examined to localize cells that produce the NPY Y1 receptor using *in situ* hybridization histochemistry. Observation of increased expression of the Y1 receptor mRNA in cells of hungry *X. laevis* would support the hypothesis that NPY mediation in olfaction may indeed involve binding to NPY receptors. Also, the *in situ* analysis would indicate which olfactory tissue produces the receptors. The previous gel analysis suggested that another NPY receptor might mediate appetite control through olfaction, the Y5 receptor. Thus, in future, this study plans to localize Y5 receptors repeating the *in situ* procedure after localization for Y1. Further studies of the NPY mechanism in amphibian models would aid in understanding regulation of appetite in more complex vertebrates such as mammals. Such knowledge would be valuable in developing pharmacological methods for addressing the serious obesity pandemic, a disease largely controlled by human feeding patterns.

A Comparative Study of Two Histone Deacetylase Inhibitors, CG-1521 and SAHA, on MCF-7 Human Breast Cancer Cells

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Histone deacetylase inhibitors (HDIs) are currently being evaluated for their therapeutic potential and have shown considerable promise as a possible adjuvant therapy for many cancer types. This study compared the activity of two HDIs, CG-1521 and SAHA, on p53+/+ MCF-7 human breast cancer cells. Crystal violet growth assays of CG-1521 and SAHA treated cells show differential levels of apoptosis with CG-1521 having a much earlier and more cytotoxic effect. Flow cytometry confirmed this, showing clear apoptosis in CG-1521 treated cells (37%) and relatively little apoptosis in SAHA treated cells (4%) after 48 hours. Quantitative RT-PCR of subsets of apoptotic and cell cycle regulatory genes reveal similar gene induction trends among CG-1521 and SAHA treated cells, however, the levels of induction differ greatly between the two treatments. Cell cycle associated genes were slightly down regulated by CG-1521 but dramatically down regulated by SAHA including Cyclin B1, cdc20, and Kntc2. Apoptotic associated genes such as p21B and Gdf15, however, were dramatically up regulated by CG-1521 and only slightly up regulated by SAHA. These differential levels of gene induction provide molecular evidence of both cell cycle arrest and apoptosis, and suggest a molecular mechanism that explains the difference in biological effect of the two HDIs. Previous studies using HDIs on LNCaP human prostate cancer cells have determined that posttranslational modifications of the tumor suppressor protein, p53, stabilize the protein, thereby affecting its downstream target gene activation. Western blot analysis shows the presence of both p53 Ac-Lys 373 and p53 Ac-Lys 382 in both CG-1521 and SAHA treated cells suggesting that the effects of the HDIs on p53 acetylation in MCF-7 cells are more complex than previously described in LNCaP cells. However, further experiments must be done to confirm these data.

The effect of nordihydroguaiaretic acid on NPC1 cells.

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The occurrence of a rare disorder known as Niemann-Pick type C disease has been linked to a mutation in the NPC1 or NPC2 gene. Progressive neurodegeneration, a hallmark of the disease, has been suggested to result from defective lipid transport within the cell; however, the mechanism of that defect is still unknown. The Vaughan lab has proposed that one component of the disease is abnormal motility of membranes containing the NPC1 protein. In this project, we tested how drugs that affect dynein-driven transport can influence the behavior of the NPC1 containing membranes. Cos-7 cells transfected with either the wild-type NPC1 gene or the mutated NPC1(I1061T) gene were treated with nordihydroguaiaretic acid (NDGA), a drug previously shown to

affect the phosphorylation state of the microtubule motor dynein-dynactin complex. The behavior of NPC1 positive membranes was then observed through live imaging. It was found that NDGA did affect the localization and motility of NPC1 containing membranes. This supports the link between dynein-driven transport and the normal function of NPC1 membranes. However, the NDGA treatment did not reverse the defective motility of mutant NPC1 membranes. Also, upon investigation of the effect of NDGA on the dynein-dynactin complex it was found that no significant localization at the microtubule organizing center occurred as suggested in previous experiments. Based on the results of this study it was determined that further exploration concerning the effects of NDGA on NPC1 positive membranes is necessary to establish whether the drug may prove to be a useful candidate in the treatment of NPC disease.

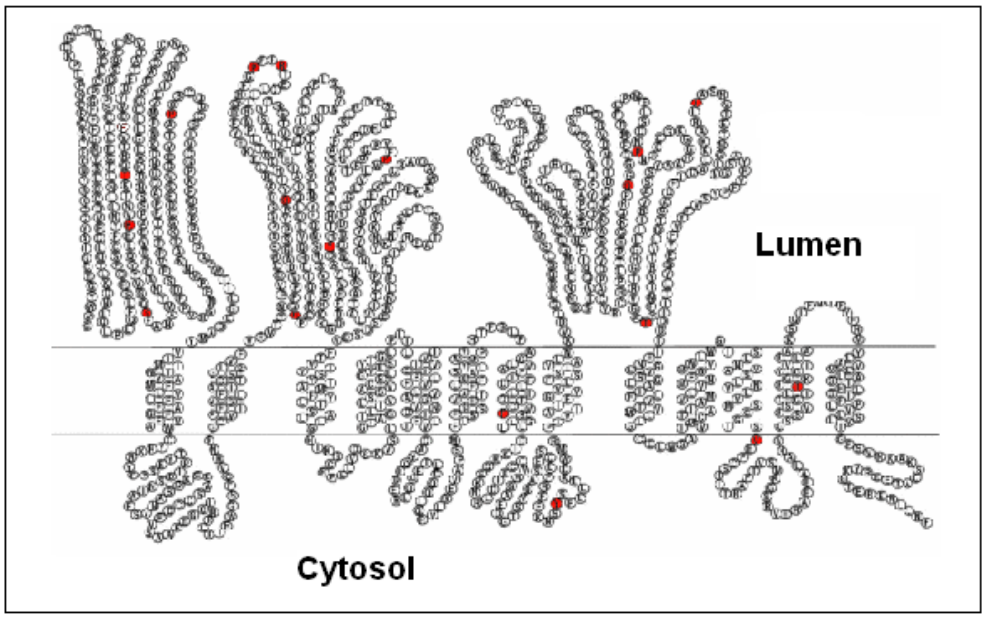

Figure 1. NPC1 transmembrane protein.

Characterizing phenotypes of mutations affecting rhodopsin trafficking

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The process of transporting rhodopsin, the primary visual protein, to the retina is poorly understood. In humans, problems with rhodopsin trafficking results in Retinitisis Pigmentosa and blindness in 1.5 million people worldwide. Several mutations identified in *Drosophila melanogaster*, labeled *KH3, KH4, KH6*, are analogous to mutations found in humans. All three KH's are recessive lethal mutations that disrupt a step in the transport of rhodopsin to the rhabdomeres of the photoreceptors. Initial deletion mapping has localized the genes to the right arm of the second chromosome. KH3 lies within the cytological region 59D1-E1, KH4 within the region 46C, and KH6 within the region 57D2-58D1. These mutations, using deletion mapping, have been mapped to

more precise regions. To analyze of the structure and organization of the photoreceptors in KH mutants, GFP labeled rhodopsin was expressed in a constant manner for pseudopupil studies. Eyes homologous for the KH mutations showed lower rhodopsin expression as determined by lower pseudopupil fluorescence. To localize rhodopsin as it migrates from the cytoplasm of the rhabdomere to the membrane, heat shock induced expression of GFP labeled rhodopsin allowed for a time course study.

Effect of Vinblastine on spindle assembly

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Vinblastine is a successful chemotherapeutic agent used in the treatment of several types of cancer including Hodgkin's lymphoma, non-Hodgkin's lymphoma, childhood acute lymphocytic leukemia, as well as breast and testicular cancer. While it is believed that Vinblastine inhibits cell proliferation at mitosis by acting on spindle microtubules, little is know about the mechanism by which this occurs. Formation of a normal bipolar spindle is essential for proper segregation of sister chromatids and formation of identical daughter cells. By examining proteins which are known to be required for normal spindle formation we hoped to elucidate the mechanism by which Vinblastine exerts its effects on the cell. We use a BSC-1 cell line expressing GFP-tagged α-tubulin with time lapse video microscopy and immunofluorescence of the proteins NuMA, dynein, dynactin, and HSET, all of which are important for normal spindle assembly. By live cell time-lapse video microscopy, we show that Vinblastine concentrations at or above 100 nM disrupt the formation of a bipolar spindle, often resulting in the formation of multipolar spindles. BSC-1 cells treated with 100 nM Vinblastine took an average of 181 minutes to complete mitosis and those treated with 300 nM Vinblastine took an average of 226 minutes as compared to 56 minutes for control cells. These cells often contained multipolar spindles and frequently fail cytokinesis. By Immunofluorescence we found that dynein and dynactin localize to the asters, while NuMA localizes to many, but not all asters. HSET, however, is found diffusely throughout the cell. Because NuMA, dynein and dynactin stabilize spindle assembly in normal cells, it is possible that these proteins play a role in aster formation in Vinblastine treated cells.