Proposal Example 1

Title and Hypothesis (1000 characters maximum):

The role of AP Endonuclease-1 in fetal development.

APE-1 has a direct effect on organ development in APE-1 HM mice.

Specific Aims (2000 characters maximum):

The expression of APE-1, and other molecules associated with oxidative stress, is increased in the placenta from humans with stressed pregnancies and fetal loss1. Since APE-1 Knock Out (KO) is embryonic lethal, its normal expression and function seems essential. By day 10 of gestation, adsorption of embryos occurs when APE-1 is inhibited2. The enlargement of the fetus requires dissemination of oxygen and nutrients which the developing cardiovascular system must serve. Thus, the development of the cardiovascular system is a critical point in fetal development so the fetus can transition to its own circulatory system. We hypothesize that it has a role in regulating the host response to oxidative stress during the development of the fetus. The goal of this project is to use MRI imaging, histopathology and assays for gene expression to evaluate embryonic loss in hypomorphic (HM) mice deficient in APE-13.

Aim 1: Determine the critical window for imaging and necropsy to evaluate embryonic loss. Using the 7 Tesla (7T) MRI for small animal research, pregnant wild-type, heterozygous and homozygous HM mice will be imaged at varying gestational ages to determine when fetuses can be quantified.

Aim 2: Quantify morphological discrepancies throughout gestation. Cohorts of pregnant dams will be necropsied at the times indicated in Aim 1 plus 2 days prior or after. The uteri will be dissected and placentation and fetuses sectioned longitudinally. Half of the tissue will be fixed for histopathology and half used in Aim 3.

Aim 3: Determine the relationship between APE-1 deficiency and fetal pathologies. Fresh tissue collected in Aim 2 will be dissected for documenting APE-1 expression by RT-PCR and western blot. The relative APE-1 expression in pathological versus developing fetuses will be compared.

Together, these studies will evaluate the morphological changes that are associated with APE-1 deficiency and lay the ground work for future studies.

Project Plan Significance (500 characters maximum):

Oxidative stress is central to inflammation, however, less is known about how it impacts fetal survival. The HM mouse provides a unique approach with which to study the role of APE-1 - a key molecule in oxidative stress. New insight into the timing and organs requiring APE-1 function during fetal development will improve strategies for more targeted manipulations in the future. Defining a role for APE-1 in organogenesis may provide insights into fetal loss that is relevant to humans and animals.

Innovation (500 characters maximum):

The innovation in this project lies in identifying a role for APE-1 in fetal loss. Further, the APE-1 hypomorphic mouse is a novel model that is just beginning to be phenotyped and characterized. The decrease in APE-1 expression provides a unique opportunity to study the role of this molecule from development to adulthood throughout life. Understanding the role for APE-1 in fetal development may lead to innovative hypotheses to explain abortion and fetal loss in multiple species.

Approach. Must include Rationale and Methods, Potential Problems and Alternatives, Experimental Rigor (statistics, validation of reagents, sample size, etc.). 8000 characters maximum.

Rationale and Methods

Rationale: Conceptual Rationale - Polymorphisms in APE-1 resulting in decreased expression or function have been associated with human diseases including cancer and neurological syndromes. A challenge to date has been the lack of an animal model. Recently, a targeted knockout mouse has been created[4]. Simultaneously, we generated a mutant in which the knockout construct included a *neo* cassette that was strategically placed to disrupt transcription. The *neo* site was flanked with *Frt* sites and removed by breeding to mice expressing FLP recombinase. The *neo-Apex1*$^{fl/fl}$ mice did indeed have a disruption in transcription (in preparation) that was restored to wild-type phenotype after removing the *neo* insert. That yielded the *Apex1*$^{fl/fl}$ mouse for targeted knockout. However, the APE-1 hypomophic (HM) mouse has never been achieved. A HM mouse has the advantage of allowing an investigator to survey the entire body to understand the function of APE-1 while targeted knockouts are biased to a tissue of interest. Our examination of HM mice has identified: 1) kinky tails; 2) extremely tough skin; 3) DNA damage in multiple tissues but especially the digestive tract and 4) a reproducible decrease in HM offspring. This leads to the unique opportunity proposed herein to identify heretofore unknown functions for APE-1 in fetal development.

Technical rationale: The mouse models were generated in collaboration with the Mouse Biology Program (MBP) at UC Davis through a collaboration between Dr. Lloyd, Dr. Ernst and other investigators associated with the project at UCSD. They generated a unique APE-1 hypomorphic mouse, which will become available to the research community so its multiple functions can be studies throughout the body. Subsequent breeding to remove the *neo* site that creates the HM phenotype generates a floxed mouse that enables tissue targeted deletion of APE-1.

The Center for Functional MRI has research instruments used for mice and other small species. The 7 Tesla instrument can resolve a 1 mm increase in the thickness of the colonic wall in mice with colitis. Its application to fetal development is new. However, should it yield data, it will provide a tool with which to study pregnant mice longitudinally. This would reduce the number of mice used and the variation that emerges when cohorts for each point in gestation are used. However, should its resolution be limited to detecting differences late in gestation, the histological assessment in Aim 2 will ensure an informative time point is not missed.
Using RT-PCR and western blots to document expression provides biologically independent assays to quantify and validate the level of APE-1 expression. The data from litter size, imaging, and histology will provide evidence for an association between APE-1 and fetal development. Future studies in which a more direct cause and effect relationship emerges would require

studies beyond the scope of this project.They could entail identifying molecular targets in affected organs and manipulating them in other ways. Nonetheless, the proposed studies are essential to justify these more comprehensive experiments.

Methods:

Aim 1: Determine the critical window for imaging and necropsy to evaluate embryonic loss.

Breeding - Neo-Apex1$^{fl/wt}$ male and female mice will be bred and expected to yield fl/fl (HM) : wt/wt : fl/wt in ratios of 1:1:2. To enhance the yield of HM offspring, *neo-Apex1*$^{fl/wt}$ females will be crossed with *neo-Apex1*$^{fl/fl}$ males to yield fl/fl : fl/wt in 1:1 ratios. All parents and offspring are genotyped, sexed, and screened for APE-1 expression in the spleen (which is an excellent predictor of APE-1 expression in all organs). Each phenotype will be enumerated. Existing and newly generated (throughout the summer program) breeding records from HM, heterozygous and wild-type mice will be compared for evidence of embryonic loss. These data are expected to document the association of APE-1 expression to embryonic loss. The heterozygous mice have an intermediate level of expression for APE-1 when compared to wildtype or HM mice. Thus, it is possible a “dose effect” may emerge.

Imaging - At 7, 10 and 12 days of gestation, pregnant dams will be anesthetized and studied by MRI to identify a time at which fetuses can be reliably counted and measured. Data are expected to detect a decrease in litter size or fetal size that will guide when to do necropsies. It is expected that 1/8 of the fetuses will appear small or being resorbed as 50% of the HM are predicted to be lost in utero.

Aim 2: Quantify morphological discrepancies throughout gestation.

Histopathology - Based on the imaging results and to bracket key times in fetal development, dams will be euthanized at 7, 10 and 12 days (unless MRI data suggest otherwise). The uteri will be dissected and fetuses sectioned longitudinally. Half of the tissue will be fixed for histopathology and half used in Aim 3. It is expected that 1/8 of the fetuses will appear small or being resorbed as 50% of the HM are predicted to be lost in utero.

Aim 3: Determine the relationship between APE-1 deficiency and fetal pathologies.

APE expression - As the spleen is of little interest, it will be dissected to screen for APE-1 expression by RT-PCR and western blot. Alternatively, sections collected for histology can be stained with antibodies detecting APE-1, and the relative expression compared using immunohistochemistry. Images will be captured on Hamamatsu digital imaging system. It is expected that HM fetuses will have an 80% decrease in APE-1 compared to WT but the absolute estimate will be tested for correlations with morphological changes in Aim 2.

Statistical analysis - As described elsewhere, the specific questions for each Aim will be addressed by analyzing the data by Chi_2 or ANOVA.

Potential problems and alternatives

The biggest problem that is anticipated is being able to make meaningful quantitative data by MRI at day 7-10 of gestation. While MRI would be ideal to provide longitudinal studies, necropsy of dams at these early time points will provide tissues that can be studied directly by histology

and for gene expression. Another challenge will be dissecting day 10 fetuses, but immunohistochemistry provides an alternative for expression studies. The lab has multiple breeding pairs set up so it is expected that sufficient numbers will be available for a meaningful study.

Experimental Rigor (statistics, validation of reagents, sample size, etc):

Existing records on litter size and genotyping will be used to estimate the impact of the relative APE-1 deficiency for a power analysis to determine the target sample size. Care will be taken to choose cohorts of sufficient size to determine the differential in progeny viability is not due to random variation in fecundity. Chi square analysis will be done to compare the frequency of the observed genotypes in the fetuses and offspring. Subsequently ANOVA will be used to examine the role of APE-1 on the sources of variation in tissue morphology, gene expression and litter size. PCR will be performed on all animals to verify that observed data is attributed to the appropriate genotype. APE-1 expression will be used to confirm that any morphological changes segregate to levels of APE-1. All primers and antibodies are checked regularly for their specificity based on the size of the molecule detected and in selected cases, sequencing.