

CURRENT RESEARCH ACTIVITIES FOR THOMAS R. HANSEN

Structure-Function of a Pregnancy-Associated Protein. The Hansen laboratory is currently in the 10th year of NIH funding for this project. A 17-kDa protein was identified by our group that was released by the endometrium in response to the developing conceptus and interferon (IFN)-tau. This protein was identified to be a ubiquitin homolog using Western blot procedures and was originally called ubiquitin cross-reactive protein or UCRP. UCRP was detectable in endometrium by day 15. It was expressed in highest amounts on days 17-18 of pregnancy, and continued to be detectable through day 35 of pregnancy. It has not been detected, or has been detected in very small amounts in endometrium from non-pregnant cows. Our group sequenced the cDNA and gene encoding bovine UCRP. Both had 30% inferred amino acid sequence identity with a tandem bovine ubiquitin repeat. Bovine UCRP, also called ISG17 and ISG15 is the ortholog of human and mouse ISG15. The bovine ISG15 gene encodes a protein of 17-kDa that migrates to an apparent molecular weight of 17,000 on PAGE gels. Human and mouse ISG15 genes encode a mature 17-kDa protein that migrates to an apparent molecular weight of 15,000 on PAGE gels. ISG15 retains LRGG C-terminal amino acids that have been described for ubiquitin to conjugate to and regulate intracellular proteins. We have demonstrated that ISG15 conjugates to uterine proteins in response to pregnancy and IFN-tau. The ISG15 mRNA is present in very low amounts in endometrium from non-pregnant cows, but is up-regulated/induced significantly in endometrium from pregnant cows. Transcription of the ISG15 gene and release of ISG15 are induced by IFN-tau activation of STATs 1-3 and IRF-1 transcription factors. Bioactive recombinant ISG15 has been developed in our laboratory that retains C-terminal Gly-Gly amino acids using baculovirus, yeast, and *E. coli* expression systems. We are currently studying extracellular bioactivity of ISG15 through several in vitro and one in vivo model. We are addressing intracellular function of ISG15 through identifying proteins that become covalently attached and targeted to degradation or regulation. Intracellular function also is being examined through immunolocalization using confocal and electron microscopy approaches. Genes targeted for regulation by the ISG15 system are being examined using microarray approaches. Likewise, the structure of ISG15 is being computer-modeled using the known structure for human ubiquitin. Finally, site-directed mutagenesis has been used to modify putative functional amino acid residues.

Initial work, using the cow as a model, identified ISG15 as an IFN-induced ubiquitin paralog that might be integral in orchestrating uterine endometrial responses to the conceptus. More recent experiments in the mouse (by our group) and in the human (by others) show that ISG15 is up-regulated in the uterus during pregnancy and possibly functions in uterine remodeling in preparation for implantation. Also, in collaboration with Dr. Dong-Er Zhang, we have shown that transgenic mice with the gene deleted for an ISG15 processing enzyme (UBP43) exhibit a fetal-lethal phenotype. The strain that we are studying has a 100% fetal mortality rate by day 17.5 of pregnancy in UBP43 *-/-* mice. We conclude from the mouse and human experiments that some functions of ISG15 might be common to all mammals and that the ISG15 system is critical for pregnancy to reach term and for fetal viability.

Early embryonic mortality occurs in 25-60% of pregnancies in mammals. This loss of embryos may be caused by dysfunctional communication between the conceptus and uterus during early pregnancy. The long-term goal of the laboratory is to increase fertility through developing biotechnologies based on study of uterine responses to the conceptus. In ruminants, IFN-tau and ISG15 probably protect the conceptus from early maternal rejection. The functions of ISG15 during early pregnancy are not known, but appear to be multifaceted in that ISG15 conjugates to proteins, but also is released by the uterine endometrium and has a cytokine/ hormonal role. It is our intent to develop strategies based on function of ISG15 that will improve pregnancy rates in females.

This project was initially funded (1994-1999) through an NIH-R29 First award and was renewed (1999-2004) through an NIH-R01 award to T.R. Hansen. We will be submitting a second five-year R01 renewal this in Spring, 2005 to the NIH. Also, a USDA grant will be submitted in a related research area wherein we have identified several differentially expressed genes in blood and uterine tissues in day 18 pregnant when compared to non-pregnant cows (see next research topic). Collaborators on this project include: Dr. James Cross (University of Calgary), Dr. Brent Bany (Southern Illinois University), Dr. James Pru, MGH-Harvard, Dr. Greg Johnson, Dr. Fuller Bazer, Dr. Tom Spencer, Texas A&M University, Dr. Art Haas, Medical College of Wisconsin, Dr. Bill Thatcher, University of Florida and Dr. Dong-Er Zhang (Scripps Institute, California).

Gene Expression in Blood and the Uterus during Early Pregnancy in the Cow. The primary focus of the research is understanding the function of pregnancy-associated proteins during maternal recognition and establishment of early pregnancy. A secondary benefit of the research is delineating blood markers for a viable day 18 embryo. Knowledge of which maternal genes and function of corresponding proteins that are up-regulated in response to the embryo should lead to biotechnologies that are designed to control reproductive efficiency and, thus, improve overall reproductive management. For example, study of blood profiles of pregnancy-associated proteins

should provide a better understanding of when and why embryos die. Likewise, knowledge of embryo-maternal interactions can be used to determine pregnancy status and facilitate more efficient estrous synchronization and re-breeding attempts.

The bovine estrous cycle is about 21 days in length and is based on the observed interval between sexual receptivity (estrus or heat). In order to achieve maximal productivity, cows must have a calf on an annual basis. The gestation length in cows averages about 283 days, so this means that re-breeding after parturition must occur within three months to stay on this annual cycle. If cows do not conceive within this time, the herd's calving interval becomes extended resulting in reduced milk production and calves with lower weaning weights. One of the major constraints to optimal production in beef and dairy industries is the inability to identify non-pregnant cows early in the breeding season. This delays the decision in re-breeding cows as much as 30 days (time when ultrasound is accurate by an experienced veterinarian). Development of an early pregnancy test would help producers make a decision on how to manage the non-pregnant cow. For example, a test, which accurately distinguishes between pregnant and non-pregnant cows on day 18 of pregnancy, would allow the producer to synchronize and artificially inseminate these cows prior to the next ovulation.

Another constraint to beef and dairy industries is the incidence of early embryo mortality. Early abortion of the embryo is a serious economic loss and is caused by a dysfunctional communication between the mother and the embryo. Feto-maternal communications are a result of chemical signals such as hormones, cytokines, or chemokines (etc.) that bind receptors and induce cellular responses. One example of this communication is the release of IFN- τ by the conceptus on days 15-18 of bovine pregnancy and the response of the maternal endometrium to this cytokine.

The beef and dairy industries contribute to most of the agricultural receipts in the State of Wyoming. One constraint to optimal productivity in these important Wyoming industries is the 30% incidence of early embryo mortality and the serious economic consequences of this failure in reproduction. It is conservatively estimated that beef and dairy industries lose \$600 million per year because of lower weaning weights and loss in milk production due to early death of the developing embryo. Basic knowledge of the mechanisms associated with establishment of pregnancy can be used to develop therapeutics or biotechnologies that are designed to improve or even control fertility in cattle. We have identified twenty-nine mRNAs that were expressed at higher levels in blood cells from day 18 pregnant when compared to non-pregnant cows using subtractive library, subtractive probe, differential screening and modified northern blot approaches. Three of these mRNAs also were found to be up-regulated in the endometrium from day 18 pregnant when compared to non-pregnant cows using northern blot. The unique attribute for these clones is that there are no corresponding known bovine sequences. Because these mRNAs are highly expressed in white blood cells and are found in greater amounts in the endometrium of day 18 pregnant when compared to non-pregnant cows, we suspect that they encode proteins that have critical function in preparing for the early developing embryo. It is hypothesized that these mRNAs encode proteins that are critical to the establishment of pregnancy. The objectives of the present experiments are to determine complete nucleotide and inferred amino acid sequence, generate recombinant protein and anti-recombinant protein antibodies, examine temporal expression of mRNAs and proteins in the uterus and blood during early pregnancy and in bovine endometrial cells treated with interferon-tau, and to determine function of these proteins in mediating establishment and maintenance of pregnancy in ruminants.

The research is currently funded through a grant (2001-present) from AspenBio, Inc (Castle Rock, Colorado) and the University of Wyoming Competitive Agricultural Grants Program (2003-2005). A revision of a research proposal was submitted to the USDA-NRI program in December, 2004.

Maternal Undernutrition Programs Fetal Heart Gene Expression. Adequate nutrient supply during early gestation is critical for fetal organogenesis. Offspring from undernourished humans and rodents have an increased incidence of obesity, diabetes, hypertension, and cardiovascular disease as adults. In general, low weight or thinness at birth is associated with increased risk of cardiovascular and metabolic disorders in later life. Preliminary experiments in sheep revealed that a global 50% nutrient restriction (protein and energy) during the first half of gestation caused compensatory growth of left and right ventricles of the fetal heart and a marked decrease in fetal weight by day 78 of gestation. Pregnant ewes were randomly grouped into control (n = 7, 100% NRC requirements and mineral-vitamin) or nutrient-restricted groups (n = 6, 50% NRC requirements and mineral-vitamin mixture). Ewes were maintained on diets from day 28 through day 78 of gestation. Fetal left ventricle (LV) was collected and snap-frozen on day 78 of gestation. Angiotensin II type 1 receptor (AT2R1) mRNA was slightly downregulated (0.7 fold; P<0.05), while matrix metalloproteinase-2 (MMP-2) mRNA was upregulated (1.7 fold; P<0.06) in fetal LV from nutrient restricted when compared to control-fed ewes. Differential screening of a fetal ovine LV subtractive cDNA library with subtractive LV cDNA probes revealed 11 differentially expressed cDNAs that were confirmed to be specific to nutrient restricted fetal heart using modified northern blot analysis. It is

concluded from these preliminary experiments that hypertrophy of fetal LV in response to maternal undernutrition is associated with differentially expressed genes. These differentially expressed genes may function during fetal heart development to regulate compensatory growth as well as remodeling of the heart, which leads to adverse cardiac function in later life. How these genes are activated and how they function in regulating hypertrophy of the fetal heart are ongoing studies that have recently been funded through a five-year (2004-2009) NIH sub-proposal to the Hansen Laboratory via the UW BRIN-INBRE programs. These experiments also are in collaboration with the newly developed UW Center for the Study of Fetal Programming. Dr. Steve Ford and Dr. Peter Nathanielsz are collaborators on this project.

Maternal and Fetal Genetic Response to Bovine Viral Diarrhea Virus Infection. A better understanding of the molecular mechanisms of bovine viral diarrhea virus (BVDV) replication, pathogenesis, and host immune responses, particularly during the establishment of persistent infection, is important for developing more efficient means of prevention, diagnosis, and treatment of cattle. This proposal tests the hypothesis that BVDV infection induces differential host gene expression in maternal and fetal blood that is distinctive in persistently infected (PI) animals. This hypothesis will be tested through the unrestricted screening of cDNA subtraction libraries derived from the blood of cows carrying PI, transiently infected (TI), and uninfected (UI) fetuses and from the blood of PI, TI, and UI fetuses harvested by C-section. Pregnant cows will be experimentally infected with non-cytopathic BVDV2 on day 75 (PI) and on day 175 (TI) of pregnancy. The specific aims include identifying, cloning, and sequencing differentially expressed genes in blood from cows carrying PI, TI, and UI fetuses and in blood from PI, TI, and UI fetuses and characterizing candidate differentially expressed genes and/or protein products. The longer-term objectives are to use differentially expressed genes as blood markers for the prenatal diagnosis of PI animals and to study the pathogenesis of BVDV infections through the study of maternal-fetal and host-viral interactions and the signaling pathways affected by BVDV infection.

This project is a collaboration with Dr. Alberto van Olphen, who recently moved from the University of Wyoming to the University of Southern Florida. Dr. Hansen and Dr. van Olphen are Co-principle investigators on this project, which was recently funded via the USDA-NRI program (2004-2007).