Project Title:

Pilot investigation of the causes of hemangiosarcoma in Clumber Spaniels[1]

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Project Period:

[09/01/2008 – 08/31/2009]

Amount Requested:

Year 1: $11,100 direct / $888 indirect
Total costs for term of grant (one year); $11,988

Signatures:

Principal Investigator _____ 4/30/08
Date

Director, Grants & Contracts Carolyn U. Witt 05/01/08
Date 4/30/08

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Scientific abstract
Although rare in humans, hemangiosarcomas (HSA) are relatively common in certain breeds of dog such as Clumber Spaniels. Significantly, many of these dogs demonstrate a familial predisposition to developing hemangiosarcomas, indicating that there is an underlying genetic component to this disease. To study these tumors we propose to establish a Canine Hereditary Cancer Consortium (CHCC). The CHCC will take advantage of new genetic resources and technologies that have been developed at the Van Andel Research Institute to develop genetic screens, diagnostic tests, and treatments for hereditary canine cancers as well as gain insight into the biology of human disease. In this pilot proposal we will focus on hemangiosarcomas in Clumber Spaniels, though later we will include other breeds and additional hereditary cancers. The specific objectives of this proposal are 1) to isolate tumor cells, DNA, and mRNA from Clumber hemangiosarcoma samples and normal tissues, 2) to perform DNA SNP array analysis of normal Clumber Spaniel tissues to identify chromosomal regions associated with disease, 3) to perform mRNA microarray analysis of hemangiosarcoma tissues to identify expression patterns that correlate with disease, and 4) to identify/validate activated, drugable tumor targets.

Lay abstract
Hemangiosarcomas are a soft-tissue tumor for which there are currently no effective treatments. Although rare in humans, hemangiosarcomas are relatively common in certain breeds of dogs such as Golden Retrievers, German Shepherds, and Clumber Spaniels. Significantly, hemangiosarcomas seem to “run” in families, indicating that there is an underlying hereditary or genetic component to this disease. Our objective is to identify that hereditary component. To do this we have formed a consortium of leading cell biologists, geneticists, computer experts, and veterinarians to take advantage of the advanced cancer technologies in several laboratories within the Van Andel Research Institute. We will analyze collected DNA and RNA samples from Clumber Spaniels for genetic patterns that are associated with this disease. These patterns may form the basis of genetic tests that can tell us whether a particular dog is a carrier of a defective gene that will cause cancer. Also, these studies may provide important clues about hemangiosarcomas in people.

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Significance of Research

There are many subtypes of cancers that occur infrequently in the population. Unfortunately, these rare or "orphaned" cancers are largely ignored in research efforts that target the more common cancers. Hemangiosarcomas are one example of an orphaned cancer. Hemangiosarcomas are a rare (2-3 cases per 10^6 population) type of soft-tissue tumor that arise from cells of endothelial origin[2]. These tumors tend to be aggressive and often multicentric, making their clinical treatment difficult. Consequently, the overall survival rate for hemangiosarcomas is low with most patients dying from metastatic disease within 2-3 years.

Although rare in humans, hemangiosarcomas are relatively common in certain breeds of dogs such as Golden Retrievers, German Shepherds, and Clumber Spaniels. Significantly, many of these dogs demonstrate a familial predisposition to developing hemangiosarcomas, indicating that there is an underlying genetic component to this disease. Dogs are a particularly good model for studying hereditary disease; they have a relatively short generation time, well documented health records, and pedigrees that may be traced back for several generations. It is also noteworthy that the physiology of the dog closely resembles that of humans so what is discovered in one species may be translated to the other. Thus, identification of the genetic lesion(s) that predisposes dogs to hemangiosarcomas will have significant repercussions for human health also.

Background of Research and Preliminary Work

To study these tumors we propose to establish a Canine Hereditary Cancer Consortium (CHCC) at the Van Andel Research Institute. The CHCC will take advantage of new genetic resources and technologies that have been developed at VARI for the study of cancer to develop genetic screens, diagnostic tests, and treatments for hereditary canine cancers as well as gain insight into the biology of human disease. Initially we will focus on hemangiosarcomas in Clumber Spaniels. In the future we would like to expand our studies to include other breeds with hemangiosarcomas as well as additional canine hereditary cancers. In this pilot study we propose to use Clumber Spaniel DNA samples that have been collected by the Clumber Spaniel Club of America (CSCA). Clumber spaniels are ideally suited for this sort of analysis because of the low-degree of heterozygosity in this breed[3]. In 1998 the CSCA embarked on an ambitious program to collect blood samples from as many Clumber Spaniels as possible to create a DNA bank. The rationale for this ongoing effort is that the samples gathered in this project will be invaluable for future Clumber-specific research projects into various heritable diseases (such as intervertebral disc disease, epilepsy, hip dysplasia, hemangiosarcoma and eye problems). Through the CSCA we have obtained access to 3 DNA samples from dogs diagnosed with

[2] Weiss, S., and Goldblum, J. (2008) *Soft Tissue Tumors*, 4th ed., Elsevier, Philadelphia.
[3] Parker, H. G., Kim, L. V., Sutter, N. B., et al. (2004) *Science* **304**, 1160-1164

HSA as well as 3 DNA samples from closely related but unaffected dogs. In addition we propose to collect additional DNA samples prospectively. Over the one year lifetime of this project we expect to collect at least 3 additional DNA samples from dogs diagnosed with HSA as well as 3 DNA samples from closely related but unaffected dogs.

The key technologies of the CHCC are broadly distributed across four laboratories at the Van Andel Research Institute. These labs have distinct yet complementary expertise so that together they form a strong and comprehensive research team. Another key member of the CHCC is Roe Froman, DVM, Chairperson of the Genetic Health Committee and former President of the Clumber Spaniel Club of America. She lends her clinical expertise and familiarity with this breed and the Clumber community.

The key technologies of this proposal are:

1) The Laboratory of Cancer and Developmental Cell Biology, run by Nicholas S. Duesbery, Ph.D., has established standardized protocols for isolating and cryopreserving sarcoma cells from human tumors. Using tissues from local hospitals as well as the Cooperative Human Tissue Network they have already extracted more than 40 sarcoma cell isolates from a broad variety of adult and childhood tumors. This laboratory will isolate and cryopreserve canine hemangiosarcoma cells and provide these cells, their DNA, and their mRNA for analysis by other laboratories. The Laboratory of Cancer and Developmental Cell Biology will also validate the biochemical signatures using banked, frozen tumor cell isolates and any appropriate methodology (RT-PCR, qPCR, immunoblotting, pharmacologic inhibition).

2) The Laboratory of Cancer Genetics and Sequencing, headed by Bin T. Teh, M.D, Ph.D., has expertise in familial cancer and array profiling of tumor samples using an Affymetrix-based system. This team is using the Affymetrix high-density (500k) human Single Nucleotide Polymorphism (SNP) array in combination with expression data to identify novel renal cell carcinoma-related genes. Dr. Teh's group will perform SNP array analyses of Clumber DNA samples using the Affymetrix Canine SNP Array (version 2, 127K SNPs) to identify genes that pre-dispose dogs to hereditary cancers.

3) The VAI Microarray Technology Core Facility, headed by James H. Resau, Ph.D., will perform gene expression analyses using Agilent canine microarrays. The VAI Microarray Technology core facility routinely assays gene expression in human and murine tissues using Agilent microarray platform. Although this core facility has previously developed spotted canine arrays (10 thousand sequences), for these studies we propose to use Agilent Canine microarrays (1 slide contains 4 arrays – each array containing 44 thousand unique sequences (genes)). The rationale for this is that Agilent

arrays offer a greater number of target sequences and quality control metrics to support the expression data.

4) The Laboratory of Computational Biology, headed by Kyle Furge, Ph.D., will analyze and integrate SNP and gene expression data and identify chromosomal markers or expression patterns of prognostic and diagnostic value. In addition they will look for expression signatures that indicate activation of a particular biochemical pathway.

Specific Objectives

The specific objectives of this proposal are:

1) to isolate tumor cells, DNA, and mRNA from canine hemangiosarcoma samples and normal tissues,

2) to perform SNP array analysis of DNA isolated from blood samples of diseased Clumber Spaniels and closely related family members to identify chromosomal regions that eventually may lead to the identification of a disease gene,

3) to perform mRNA microarray analysis of hemangiosarcoma tissues to identify an expression pattern or signatures that correlate with disease and may eventually may lead to the identification of a disease pathway, and

4) to identify and validate activated, drugable tumor targets that eventually may lead to a disease therapy.

Description of Research Design

The unique resources at VARI, including an established sarcoma research group, a hereditary cancer group, and microarray and bioinformatics cores as well as a strong collaborative association with a local veterinarian and Clumber Spaniel Club of America member provides the necessary basis for establishing the CHCC. In this pilot study we propose a focused study of hemangiosarcomas in Clumber Spaniels. Through the Clumber Spaniel Club of America we have access to 6 DNA samples isolated from normal and afflicted Clumber spaniels. We will begin our study with a retrospective SNP analysis of these samples to identify chromosomal regions that co-segregate with hemangiosarcoma. We will also collect fresh tumor samples and normal tissues prospectively. Some of these tissues will be obtained through a local veterinary clinic and while others will be come from Clumber owners recruited through the CSCA. These will be used to make cell isolates for use in tissue culture analysis as well as mRNA and DNA for gene expression studies and SNP analysis, respectively.

Although the primary focus of this application is on hemangiosarcomas, we are aware that other hereditary conditions afflict Clumber Spaniels including

intervertebral disc disease, epilepsy, hip dysplasia and other cancers. Where possible we will re-analyze SNP data with these conditions in mind. We will also begin collecting these tissues in order to establish a collection of DNA and mRNA so that when a sufficient number of cases are accumulated we can initiate an investigation of that condition.

Oversight and Personnel

Administration of the project will be coordinated through The Laboratory of Cancer and Developmental Cell Biology. Scientific oversight will be provided by a CHCC committee consisting of the principal investigators and veterinarians associated with the above laboratories. Funding for their salaries is not requested. Funding for a trained technician who will support this project will be provided by VAI through the Laboratory of Cancer and Developmental Cell Biology. Funding for a summer undergraduate (pre-Veterinary) student who will assist in this pilot project has been generously provided to the Laboratory of Cancer and Developmental Cell Biology through the Frederik and Lena Meijer Summer Research Internship Program.

Expected Outcome

AKC support we will allow us to provide 1) genetic or biochemical leads that may be translated for clinical use in order to provide evidence-based information for breeding decisions that will help to eliminate hereditary hemangiosarcoma in Clumber Spaniels and possibly other breeds of dog, 2) identification of proteins or biochemical pathways that may serve as therapeutic targets, and 3) a template for further successful investigation of canine hereditary cancers. It is our hope that successful completion of this project will lead to improvement in our understanding of the biology of HSA in dogs and perhaps in human angiosarcoma as well.

Anticipated Problems or Obstacles

Each of the methods of investigation that we have described is a powerful analytic tool in its own right. However, each also has shortcomings that may limit their utility. One of the major strengths of this proposal is that our joint pursuit of genetic, molecular, and cellular levels approaches can compensate for these deficiencies and enable a more comprehensive analytical approach. The following are examples of problems we may encounter in the course of this work.

1) The ready availability of fresh tissues and tumors from the Clumber Spaniel owners and veterinarians will be an important factor in this effort. We will promote interaction with Clumber Spaniel owners and veterinarians through Dr. Froman's position as Chair of the Genetic Health Committee of the Clumber Spaniel Club of America and President of the Clumber Spaniel Health Foundation.
2) Over the last 5 years the Laboratory of Cancer and Developmental Cell Biology has gained considerable hands-on experience isolating tumor cells from surgical samples. However we and others have frequently

noted that as tissue isolates age, a subset of cells including the large, pleiomorphic cells possessing abnormal mitoses that predominate early after cell isolation, die out or are overgrown by smaller fibroblast-like cells. These observations clearly illustrate that *in vitro* growth conditions do not accurately mimic the *in vivo* growth environment. We and others have hypothesized that important growth factors that are present *in vivo* are absent from tissue culture systems. We have found that supplementing growth medium with hepatocyte growth factor (HGF), the ligand for the receptor tyrosine kinase Met (http://www.vai.org/met/), can promote tumor cell survival *in vitro* and that HGF-conditioned cell isolates grow more robustly as xenograft tumors in athymic mice. For these reasons we will focus our investigations on early passage cells grown in the presence of HGF and endothelial cell growth supplement (bovine hypothalmic protein extract; Millipore).

3) Over the last 3 years, the Laboratory of Cancer Genetics has performed SNP analysis of hundreds of tumor samples so we do not anticipate any problems in the generation of high-quality genotype information. Identification of a discrete genomic region that confers an associated risk for the development of hemangiosarcoma could be confounded by the genetic complexity of this inherited trait. Provided a 10-fold increased risk for hemangiosarcoma incidence is maintained as additional Clumber Spaniel are included in the DNA bank, SNP analysis has a strong potential to identify a region associated with this trait in our sample population[4]. However, as a relatively low number of samples exist in this study for analysis of a suitably complex trait, there remains a risk that a single discrete region will not be identified but rather multiple regions could be implicated. If multiple regions are identified, we can overcome this problem by integrating the SNP results with the results of the gene expression analysis. If several risk-associated loci are identified, then we will focus on a region that is also implicated by the gene expression analysis. For example, if the gene expression analysis reveals a particular biochemical pathway is deregulated in hemangiosarcoma and a gene that is associated with that same biochemical pathway is located within a genomic region that is associated with risk of hemangionsarcoma development, then we will focus our efforts on this particular genomic region. As such, integration of the gene expression data will allow us to empirically increase the probability that a discrete genomic region will be identified.

4) Over the last 8 years, the Laboratories of Cancer Genetics, Microarray Technology, and Computational Biology have successfully generated and analyzed gene expression data produced from tumor derived specimens and we anticipate no problems in this regard. The quality of the RNA is the

[4] Lindblad-Toh, K., Wade, C. M., Mikkelsen, T. S. et al. (2005) *Nature* **438**, 803-819. Karlsson, E. K., Baranowska, I., Wade, C. M., et al. (2007) *Nat Genet* **39**, 1321-1328.

most important variable for the successful completion of this objective. To prevent problems with the generation of gene expression data, the quality of the RNA will be extensively analyzed before application to the gene expression microarrays A second obstacle in the analysis of microarray data is that often individual genes that are identified as misregulated in the tumor represent genes involved in cellular division. As such, this class of genes likely represents the effect of tumor growth and does not indicate the underlying cause of tumor growth. To compensate for this, in addition to analyzing global changes in gene expression we will analyze the expression of genes that are associated with deregulation of pathways that are known to directly associate with tumor development. To do this we will "translate" human gene expression pathways into canine-equivalent data. This pathway-specific analysis will partially alleviate over-interpretation of the gene expression data. Finally, we will have to select the control tissues as "normal" to compare with hemangiosarcoma. We may use universal (pooled) canine mRNA or resort to laser capture microscopy or flow cytometry to isolate normal endothelial cells from Clumber tissues.

5) Nanoscale technology is currently used by microarray-based methods to measure the expression levels of thousands of genes simultaneously and to measure thousands of single-nucleotide polymorphisms (SNPs) simultaneously. The nanoscale nature of these microarray-based approaches means that each measurement will contain a certain amount of inherent experimental noise. As such, there is a trade-off between the number of expression/SNP measurements taken and the accumulation of experimental noise that is found with nanoscale devices. While a spectrum of statistical methods, such as variance stabilization and moderated test statistics have been developed to partially compensate for the experimental noise, a certain level of uncertainty still exists for each individual gene expression or SNP measurement. The uncertainty in these measurements can be quantified as the false-discovery rate (FDR). The FDR is expressed as a percentage in which the measurement is the result of random experimental noise. While one way to decrease the FDR is to increase the number of samples analyzed, an alternative method to control for FDR is to use the microarray datasets to cross validate each other.

6) Cellular analyses allow direct measurement of protein levels and activity. They are an indispensable analytic tool that can confirm microarray results and extend their findings to promote a mechanistic understanding of altered cellular response. Despite the high degree of conservation between mammalian species, some of the reagents that are available for cellular analysis for more popular animal models such as mice and humans, may not work well in dogs. However, the availability of canine SNP arrays, microarrays, and the canine genomic sequence will facilitate primer design and cloning efforts and allow us to by-pass these hurdles.

Also, VAI has a Monoclonal Antibody Production Core that can custom make monoclonal antibodies against peptides or recombinant proteins.

Biographical profiles.

Nicholas S. Duesbery, Ph.D. Principal Investigator (5% effort) Dr. Duesbery received both his M.Sc. (1990) and Ph.D. (1996) degrees in zoology from the University of Toronto, Canada, under the supervision of Yoshio Masui. Before his appointment as a Scientific Investigator at VARI in April 1999, he was a postdoctoral fellow in the laboratory of George Vande Woude in the Molecular Oncology Section of the Advanced BioScience Laboratories–Basic Research Program at the National Cancer Institute–Frederick Cancer Research and Development Center, Maryland. Dr. Duesbery was appointed Deputy Director for Research Operations in March 2006.

Roe Froman, DVM. Co-Investigator (2.5% effort) Dr. Froman obtained her DVM (1990) from Michigan State University. Following this she performed a preceptorship at the National Zoo in Washington, DC. Before her appoinment as a Veterinarian at Northeast Cat and Dog Hospital in 1995 she was an Associate Veterinarian and Zoo Veterinarian at the Animal Clinic, PC, in Grand Rapids, MI. She is an active member of the American Veterinary Medical Association and President of the Clumber Spaniel Health Foundation as well as Chair of the Genetic Health Committee and Past President of the Clumber Spaniel Club of America.

Kyle Furge, Ph.D. Co-Investigator (2.5% effort) Dr. Furge received his Ph.D. in biochemistry from the Vanderbilt University School of Medicine in 2000. Prior to obtaining his degree, he worked as a software engineer at YSI, Inc., where he wrote operating systems for embedded computer devices. Dr. Furge did his postdoctoral work in the laboratory of Dr. George Vande Woude. He became a Bioinformatics Scientist at VARI in June of 2001 and a Scientific Investigator in May of 2005.

James H. Resau, Ph.D. Co-Investigator (2.5% effort) Dr. Resau received his Ph.D. from the University of Maryland School of Medicine in 1985. He has been involved in clinical and basic science imaging and pathology-related research since 1972. Between 1968 and 1994, he was in the U.S. Army (active duty and reserve assignments) and served in Vietnam. From 1985 until 1992, Dr. Resau was a tenured faculty member at the University of Maryland School of Medicine, Department of Pathology. Dr. Resau was the Director of the Analytical, Cellular and Molecular Microscopy Laboratory in the Advanced BioScience Laboratories–Basic Research Program at the National Cancer Institute–Frederick Cancer Research and Development Center, Maryland, from 1992 to 1999. He joined VARI as a Special Program Senior Scientific Investigator in June 1999 and in 2003 was promoted to Deputy Director. In 2004, Dr. Resau assumed as well the direction of the Laboratory of Microarray Technology to consolidate the imaging and quantification of clinical samples in a CLIA-type research laboratory program. In 2005, Dr. Resau was made the Division Director of the quantitative

laboratories (computational biology, epidemiology, microarray, pathology-histology, and proteomics).

Bin T. Teh, M.D., Ph.D. Co-Investigator (2.5% effort) Dr. Teh obtained his M.D. from the University of Queensland, Australia, in 1992, and his Ph.D. from the Karolinska Institute, Sweden, in 1997. Before joining the Van Andel Research Institute (VARI), he was an Associate Professor of medical genetics at the Karolinska Institute. Dr. Teh joined VARI as a Senior Scientific Investigator in January 2000. Dr. Teh's research mainly focuses on kidney cancer, and he is currently on the Medical Advisory Board of the Kidney Cancer Association. He was promoted to Distinguished Scientific Investigator in 2005.

Scientific articles that were recently published by CHCC members.

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Detailed Budget

Cost estimates are provided on the assumption that we will process 6 archived DNA samples and 6 fresh samples (3 tumor, 3 matched normal). Per sample costs are also included.

Service	per sample	Year 1
Sarcoma cell isolation and cryopreservation	450.00 X 6	2700.00
SNP services and supplies	400.00 X12	4800.00
Microarray services and supplies	400.00 X6	2400.00
Bioinformatics services	100.00 X12	1200.00
Total	1,350.00	11,100.00
	Total Direct Costs	11,100.00
	Indirect Costs (8%)	888.00
	Total Costs	11,988.00

Timeline

Anticipated dates for the completion of specific objectives and intermediate project milestones are,

1) to isolate tumor cells, DNA, and mRNA from canine hemangiosarcoma samples and normal tissues; *over the course of the one year project we expect to obtain 3 fresh hemangiosarcoma tissues (plus 3 matching normal adjacent tissues). As we receive these tissues we will isolate tumor cells, DNA, and mRNA.*

2) to perform SNP array analysis of DNA isolated from blood samples of diseased Clumber Spaniels and closely related family members to identify chromosomal regions that eventually may lead to the identification of a disease gene; *we will have performed all SNP array analysis of archived normal Clumber Spaniel DNA in the first 3 months. Data analysis will be ongoing but may be expected to be complete by the end of 6 months. SNP analysis of samples collected prospectively will occur throughout the year as these samples become available.*

3) to perform mRNA microarray analysis of hemangiosarcoma tissues to identify an expression pattern or signatures that correlate with disease and may eventually may lead to the identification of a disease pathway; *microarray analysis of hemangiosarcoma tissues collected prospectively will occur throughout the year as these samples become available.*

4) to identify and validate activated, drugable tumor targets that eventually may lead to a disease therapy; *by the end of the project we expect to have in hand genetic or biochemical data that may be adapted for clinical use and may provide evidence-based information for breeding decisions to eliminate hereditary cancers in Clumber Spaniels and possibly other breeds of dog. Further, upon completion of this small scale project we will have established a multi-disciplinary experimental approach for identifying and validating genetic and biochemical markers of hereditary disease in dogs.*

Current Funding Sources on Research-Related Topics

R01CA109308 (Duesbery, N.) 07/07/04 – 04/30/08
DHHS/NIH/National Cancer Institute
MEK Signaling in Sarcoma Growth and Vascularization
The major goal of this project is to define the role of MEK signaling in growth and vascularization of human sarcoma and determine whether inhibition of multiple MEKs by agents such as LeTx may form the basis of a novel and innovative therapeutic approach in the treatment of human sarcoma.
Role: Principal Investigator

R01CA109308 (Duesbery, N.) 09/13/05 – 04/30/08
DHHS/NIH/National Cancer Institute
MEK Signaling in Sarcoma Growth and Vascularization
The major goal of this project is to supplement the research project with a underrepresented minority trainee.
Role: Principal Investigator/Mentor

Pending Funding Sources for Research on Proposed Topic and on Related Topics

2R01CA109308 (Duesbery, N.) 01/01/09 – 12/31/13
DHHS/NIH/National Cancer Institute
MEK Signaling in Sarcoma Growth and Vascularization
The major goal of this project is to define the individual roles of MKK in vascularization and angiogenesis of human sarcoma.
Role: Principal Investigator

(Duesbery, N.) 09/01/08 – 08/31/09
Elsa Pardee Foundation
Tumor endothelial response to MKK inhibition.
The major goal of this project is to use *in vivo* approaches including high resolution ultrasound, fluorescence, and electron microscopy to characterize the response of sarcoma associated endothelial cells to loss of MKK activity.

(Duesbery, N.) 09/01/08 – 08/31/09
MidWest Eye-Banks
MAPK activation during retinopathy of prematurity.
The major goal of this project is to use in vivo approaches to investigate the role of MAPK during phases of hyperoxia-induced vaso-obliteration and hypoxia-induced neovascularization that occur in retinopathy of prematurity.