GENETICS OF X-LINKED AND AUTOSOMAL RECESSIVE HEREDITARY NEPHROPATHY IN THE DOMESTIC DOG

A Dissertation

by

REBECCA JANE BELL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Veterinary Microbiology

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Approved by:

Chair of Committee,	Keith E. Murphy
Committee Members,	George E. Lees
	Charles Long
	James Womack
Head of Department,	Gerald Bratton

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ABSTRACT

Genetics of X-linked and Autosomal Recessive Hereditary Nephropathy in the Domestic Dog. (December 2007)

Rebecca Jane Bell, B.S., Baylor University

Chair of Advisory Committee: Dr. Keith E. Murphy

Although typically thought of as a beloved companion or indispensable aide, the domestic dog (*Canis lupus familiaris*) has emerged as an excellent model for the study of human hereditary diseases. Many hereditary diseases of the dog have nearly identical clinical presentations as those of the human and are, most often, caused by mutations in the same genes. One such disease is hereditary nephropathy; an inherited glomerular disease in the domestic dog that is similar to Alport syndrome of the human. Both diseases are caused by mutations in the type IV collagens genes, and the disease has nearly identical pathology and clinical presentations in the dog and human. By studying this disease in the dog, our laboratory hopes to increase understanding of the disease so that information that can be applied to both the human and the dog. Reported here is 1) the development of a genomic based test to determine genotypes of mixed breed dogs in a colony presenting with X-linked hereditary nephropathy, 2) the determination of patterns of X-chromosome inactivation in normal dogs and dogs that are carriers of X-linked hereditary nephropathy, 3) the design of a synthetic *COL4A5* cDNA to be used for gene therapy treatment of dogs with X-linked hereditary nephropathy, 4) the

investigation of type IV collagen gene expression changes in normal dogs and those affected with X-linked and autosomal recessive hereditary nephropathy, and 5) the discovery of the mutation causative for autosomal recessive hereditary nephropathy in the English Cocker Spaniel. Utilization of the colony of dogs affected with X-linked hereditary nephropathy (for which the causative mutation was previously identified) allowed for comparisons of type IV collagen gene expression to English Cocker Spaniels with autosomal recessive hereditary nephropathy. These data were critical to identification of the gene harboring the causative mutation for autosomal recessive hereditary nephropathy. Sequencing was performed to identify the mutation. With the ability to test for carriers of this disease, it is our hope that breeders will use it to to maintain the desired traits in the ECS while simultaneously eliminating the production of affected offspring.

DEDICATION

For Clayton

ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Keith Murphy, for giving me my first opportunity to work in a lab and for allowing me to continue my work as a doctoral student. The opportunities he provided to me have helped me to develop as a scientist, and the skills I have learned are invaluable. Without his support and guidance, I would not be where I am today. I would also like to thank Dr. George Lees for his patient tutelage in all things renal. His enthusiasm is infectious and has inspired me to pursue postdoctoral work that concerns the kidney. Also, to Dr. James Womack and Dr. Charles Long, thank you for your encouragement and assistance through my graduate career. I appreciate the time you have taken to help me accomplish this goal. I must also thank our collaborator, Dr. Clifford Kashtan, for his generosity in time and laboratory equipment.

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CHAPTER I

INTRODUCTION

The dog as a model

For many years, the mouse has been a workhorse of biomedical and genetic research. Although this organism has provided invaluable information about diseases and development, the dog, *Canis lupus familiaris*, is emerging as an equally important and perhaps more useful model of human hereditary diseases. Throughout history, the dog has proven to be a dedicated companion and tireless worker. Now, it has become an invaluable research model. It is, in fact, the unique relationship we have with our dogs that allows us to utilize them in this way.

Recent work indicates that that dogs were first domesticated from grey wolves (Vila et al., 1997; Savolainen et al., 2002) in East Asia. However, archeological and mitochondrial data disagree on the dates of this occurrance. Studies using mitochondrial DNA suggest that domestication occurred anywhere from 40,000 to 15,000 years ago and archeological data suggest domestication at 14,000 to 9,000 years ago (Leonard et al., 2002; Savolainen et al., 2002). Through thousands of years of controlled breeding, humans were able to select for the characteristics they found most valuable. Some dogs were chosen for their speed, others for their herding abilities and still others for their retention of neotenous features. These animals eventually evolved into dogs such as the Saluki, German Shepherd Dog and Pomeranian.

This dissertation follows the style and format of Gene.

Today, there are more than 350 different breeds of dog, with the majority having been created in the past 250 years (Ostrander and Giniger, 1997). The phenotypic diversity of these breeds is staggering; among mammals, the dog is the most phenotypically diverse species alive today. In order to obtain and maintain the desired traits, much inbreeding within breeds has occurred. This has fixed desired behavioral and physical traits in a breed, but also resulted in selection of undesirable genetic defects and diseases. There are more than 450 known hereditary diseases of the dog, over half of which are also found in the human (Ostrander and Giniger, 1997). These diseases often have similar, if not identical, clinical presentations in both the human and dog, and many are caused by mutations in the same genes (Ostrander and Giniger, 1997).

The fact that canine hereditary diseases occur naturally, unlike those in the majority of murine models, makes the dog an extraordinary model of human diseases. In addition, the dog is more closely related to the human than is the mouse in both size and genetic make-up (Kirkness et al., 2003). The reduced heterogeneity, short generation times, large litters, and extensive pedigree data are also of benefit to researchers. Because dogs share our homes, exercise habits and food (sometimes), the contribution these factors play in the development and outcome of diseases can be more accurately studied. In addition, in the United States, dogs are provided with a level of health care second only to the human (Ostrander et al., 2000). Therefore, extensive medical records that describe symptoms, pathology and treatment of both infectious and hereditary diseases are available.

The canine genome

Highlighting its utility as a model organism, the dog was selecetd to have its genome sequence. It was the fourth mammalian genome to be sequenced. The first sequence of the canine was completed in 2002 genome, by Celera Genomics. Celera. reported a 1.5X sequence of a male standard poodle and this sequence provided 77% coverage and was done using a shotgun approach (Kirkness et al., 2003). Unfortunately, this sequence was not made publicly available. Therefore, the National Human Genome Research Institute (NHGRI) produced a 7.8X sequence in 2004 (Lindblad-Toh et al., 2005). Sequencing was performed using DNA from a female boxer due to her high degree of genetic homogeneity with dogs representing many breeds. This sequence provides >95% coverage and is available for public use at www.ncbi.nih.gov/genome/guide/dog (Lindblad-Toh et al., 2005). The availability of these sequences has greatly advanced the field of canine genetics, helping researchers to utilize the dog to its fullest extent as a model organism.

Biology of the glomerular basement membrane

The main functional unit of the kidney is the nephron in which urine is formed. Each nephron consists of two main components, the glomerulus and the tubules. Respectively, these compartments serve to filter the blood plasma and to produce and refine the urine. To make the filtrate that will subsequently be processed by the tubules, the plasma is filtered by a selective barrier in the glomerulus consisting of 1) the fenestrated endothelium, 2) the glomerular basement membrane (GBM) and 3) the

epithelial cells (podocytes). For the purposes of this dissertation, only the GBM will be discussed in further detail.

The GBM functions as a filtration barrier and, like other basement membranes, as a site for cell adhesion and migration. Both the endothelial cells and podocytes contribute to the formation of the GBM (Abrahamson, 1987). The GBM is 300-350nm thick and composed of three distinct layers: 1) the lamina rare interna, 2) the lamina densa and 3) the lamina rara externa (McCarthy, 1997). This specialized basement membrane is comprised of type IV collagen, laminin, fibronectin, entactin/nidogen, heparan sulfate proteoglycans and, in lesser amounts, types III and V collagen (Miner, 1998). This complex protein network allows the passage of small molecules, but restricts the passage of molecules larger than albumin. The negatively charged proteoglycans are thought to contribute to filtration by blocking the passage of negatively charged plasma proteins (Miner, 1998).

Type IV collagens

The type IV collagens are important components of the GBM. The network of type IV collagens provides the framework onto which the other GBM components are attached. There are six type IV collagen alpha chains: α1(IV)-α6(IV), all of which have a similar genomic arrangement and protein structure.

The genes encoding the type IV collagens (*COL4A1-COL4A6)* are arranged in pairs, in a head-to-head fashion. *COL4A1* and *COL4A2* are found on *Homo sapien* chromosome 13 (HSA13) and *Canis familiaris* chromosome 22 (CFA22), *COL4A3* and

COL4A4 are found on HSA02 and CFA25 and, finally, *COL4A5* and *COL4A6* are found on the X-chromosome in both the dog and human (Griffin et al., 1987; Boyd et al., 1988; Hostikka et al., 1990; Mariyama et al., 1992; Sugimoto et al., 1994; Momota et al., 1998; Lowe et al., 2003; Wiersma et al., 2005). *COL4A1* and *COL4A2* are co-expressed and their protein products co-localize and the same is true for the *COL4A3*/*COL4A4* gene pair (Poschl et al., 1988; Timpl, 1989). Transcription of the *COL4A5*/*COL4A6* gene pair is not well understood, and unlike the other type IV collagens, these genes are not always co-expressed (Ninomiya et al., 1995). That is, *COL4A5* may be expressed in the absence of *COL4A6*.

The type IV collagens are approximately 1700 amino acids in length and each is divided into three segments: 1) the N-terminal 7S domain, 2) the collagenous domain and 3) the C-terminal non-collagenous, or NC1, domain. The collagenous domain has a repetitive Gly-X-Y sequence of approximately 1400 amino acids; X and Y are most commonly proline or hydroxyproline. Interruptions exist in this repetitive sequence, and allow the molecule flexibility.

To form a functional collagen molecule, three type IV collagen monomers will assemble to form a heterotrimer. The three NC1 domains will assemble, and the collagenous domains will form a right-handed triple helix from the carboxy to the animo-terminus (Boutaud et al., 2000). The proper formation of the heterotrimer is essential to basement membrane function. When properly formed, these heterotrimers will assemble to form a network by bringing two NC1 trimers together to form a dimer, and bringing four 7S domains together to form a tetramer (Hudson et al., 1993).

Although many combinations could theoretically be made, the six type IV collagen monomers will trimerize to form only three heterotrimers: α1.α1.α2(IV), α3.α4.α5(IV) and α5.α5.α6(IV) (Timpl, 1989; Gunwar et al., 1998; Sado et al., 1998). The α1.α1.α2(IV) heterotrimer is found ubiquitously in basement membranes (Timpl, 1989). The α3.α4.α5(IV) heterotrimer is found in the GBM of the adult kidney, lung, testis, cochlea and eye (Butkowski et al., 1989; Kleppel et al., 1989; Sanes et al., 1990; Miner and Sanes, 1994). Finally, the α5.α5.α6(IV) heterotrimer is found in skin, smooth muscle, esophagus and Bowman's capsule (Peissel et al., 1995; Borza et al., 2001). The disruption or absence of the α3.α4.α5(IV) heterotrimer causes Alport syndrome and hereditary nephropathy.

Alport syndrome and hereditary nephropathy

Clinical aspects

Alport syndrome (AS) and hereditary nephropathy (HN) refer to any of the inherited glomerular nephropathies caused by mutations in the three members of the type IV collagen gene family that are required for normal structure and function of the GBM. These diseases occur in the human (AS) and naturally in various breeds of the domestic dog (HN). Although this disease is not highly prevalent in the human population (1/50000 births), it is the second most common genetic cause of renal failure (2006). Both AS and HN are progressive diseases and patients inevitably progress to end-stage renal disease (ESRD) and the only treatment options currently available for the human are dialysis and renal transplantation.

Although the genetic causes of AS and HN are diverse, the primary structural abnormalities common to all forms of the disease are defects in the GBM. Secondary organ systems such as the cochlea and eye may also be affected to differing degrees (Kashtan, 1998). The kidney, however, is the primary affected organ.

The abnormalities in the AS and HN affected kidney include a distinctive multilaminar splitting of the GBM, which contributes to the development of hematuria, proteinuria and progressive renal injury. Due to the lack of a functional α3.α4.α5(IV) heterotrimer in the GBM, plasma cannot be properly filtered. Damage to the glomerulus increases as proteins traverse the GBM and enter the Bowman's capsule. These proteins are eventually excreted in the urine. Proteinuria begins as microalbuminuira and increases in severity because more damage to the GBM allows both larger volumes of protein and proteins of larger molecular weight to filter through the GBM, which the proximal tubules are unable to reabsorb properly. The presence of these proteins in the tubules can induce an inflammatory response that may lead to the infiltration of both lymphocytic and histiocytic cells and cause patchy fibrosis. This insult increases the damage to the entire nephron and eventually renders it nonfunctional. As more nephrons are lost, the survivors are forced to increase their workloads in order to maintain extracellular fluid homeostasis. The glomeruli in these nephrons, however, also have an abnormal GBM and due to the increased workload, damage to both the glomerulus and tubules progresses at an increased rate. Once the surviving nephrons are too few in number, the patient suffers renal failure. Therefore, it is the accumulation of structural and functional damage to the entire kidney that leads to ESRD.

Genetics

Both AS and HN are caused by mutations in the type IV collagen genes, specifically *COL4A3*, *COL4A4* and *COL4A5*. As previously written, the protein products of these three genes form the α3.α4.α5(IV) heterotrimer that localizes to the GBM. AS and HN can be transmitted in autosomal recessive, autosomal dominant and X-linked fashions.

The majority of AS and HN cases (>85%) are transmitted in an X-linked fashion (Kashtan, 1998). These cases are due to mutations occurring in the *COL4A5* gene, which is located on both the human and canine X-chromosomes (Hostikka et al., 1990; Sugimoto et al., 1994). Males affected with this disease will develop proteinuria early in life, progressing to ESRD early in the second decade of life. Females will be heterozygous for the causative mutation and will progress at various rates, possibly due (in part) to different patterns of X-chromosome inactivation (Vetrie et al., 1992). The remaining ~15% of cases are autosomal recessive and occur due to mutations in either the *COL4A3* or *COL4A4* genes, located on HSA02 and CFA25 (Lowe et al., 2003; Wiersma et al., 2005). There have been few documented cases of autosomal dominant AS and HN, also due to mutations in *COL4A3* or *COL4A4* (Pescucci et al., 2004).

Animal models

Currently, there exist three animal models of X-linked AS and HN, two of which are naturally occurring canine models and one of which is a “knock-in” murine model. The first canine model discovered was the Samoyed model in 1977 (Bernards and Valli,

1977). These dogs transmit HN in an X-linked fashion (XLHN) with pathology similar to that seen in human XLAS. These dogs exhibit increasing proteinuria and enter ESRD at an average of eight to ten months of age (Jansen et al., 1987). Staining for the α3(IV), α4(IV) and α5(IV) proteins in the GBM revealed a complete absence of this heterotrimer (Thorner et al., 1989). Unlike many human cases of XLAS, however, these dogs do not exhibit any of the extra-renal manifestations such as ocular abnormalities or sensorineural deafness (Thorner et al., 1988). Therefore, the most accurate term for this disease is HN. Further work revealed that HN in this Samoyed model is due to a nonsense mutation in exon 35 of *COL4A5* (Zheng et al., 1994).

The second canine model was documented in 1993 by Dr. George E. Lees of Texas A&M University (Lees et al., 1999). These dogs were brought to the Small Animal Clinic at Texas A&M when some of the males in a family developed adolescent renal failure. The clinical, pathological and immunohistochemical findings were consistent with human XLAS (Lees et al., 1999). As in human XLAS and the XLHN Samoyed model, there was a lack of staining for α3(IV), α4(IV) and α5(IV) proteins in the GBM of affected individuals (Lees et al., 1999). Also like the Samoyed model, these dogs lack any auditory or ocular abnormalities. In 1998 Dr. Lees established a colony of dogs that present with XLHN using semen from two of the originally affected males. This colony was used to further characterize this disease. The colony contains normal dogs of both genders, XLHN-affected males and XLHN-carrier females. Sequencing of the coding region of *COL4A5* revealed a ten base pair deletion in exon nine and this

causes a frameshift leading to a premature stop codon in exon ten (Cox et al., 2003). These dogs are termed NAV dogs, due to their city of origin, Navasota, Texas.

The affected males in this colony initially show proteinuria at three to four months of age and reach ESRD between ten and fifteen months of age (Lees et al., 1999). Female carriers have a much longer life-span compared to the affected male NAV dogs. The carriers become proteinuric at approximately four months of age; this proteinuria will increase until about 12 months of age. At this point, the proteinuria is stable and the dogs may live a long life with acceptable renal function, a phenomenon that is not well understood.

In 2004 the third model of XLAS, a murine model, was described by Rheault et al. (Rheault et al., 2004). This model was produced by the introduction of a nonsense mutation into the first exon of *COL4A5* (Rheault et al., 2004). Affected males show an absence of α5(IV) staining in the GBM and die between six and 34 weeks of age. Female carriers show a mosaic pattern of staining for α5(IV) and die at eight to 45 weeks of age. These animals show a pattern of disease progression similar to that seen in humans and are, therefore, an animal model of this disease.

In addition to the canine models of XLHN, there also exists a naturally occurring canine model of autosomal recessive HN (ARHN). This disease was first documented approximately 50 years ago by Dr. Krook at the Royal Veterinary College in Stockholm Sweden (Krook, 1957). He described Cocker Spaniels presenting with renal cortical hypoplasia. This term was later refined to familiar nephropathy and later again to hereditary nephropathy. In 1992 when an English Cocker Spaniel (ECS) presented with

distinctive GBM ultrastructural changes at Texas A&M University, a systematic study of this disease was undertaken by Dr. Lees (Lees et al., 1997). Because this disease was clearly inherited in an autosomal recessive fashion, *COL4A3* and *COL4A4* were the two candidate genes. A nonsense mutation was found in exon three of *COL4A4* is causative for the disease (Davidson et al., 2007).

Three murine models of autosomal recessive AS (ARAS) have also been produced. Two of these have knockouts in *COL4A3*, and the third has knockouts in *COL4A3* and *COL4A4* (Cosgrove et al., 1996; Miner and Sanes, 1996; Lu et al., 1999). All three models have clinical phenotypes similar to that of humans with ARAS and all homozygous mutants lack the α3.α4.α5(IV) heterotrimer in the GBM.

Specific objectives

The first objective of this work was to develop a genomic based test to accurately determine the genotype of mixed breed dogs in a colony that has members with XLHN. This test was developed using the publicly available canine genome sequence and the implementation of this test will allows for planning of ongoing studies. The second objective was to design a canine *COL4A5* cDNA to be used for a gene transfer treatment of XLHN. The coding sequence of this gene was modified to suit our requirements and synthesized by a private company. The third objective of this work was to examine X-chromosome inactivation patterns in female carriers of XLHN. A methylation sensitive PCR assay was used to determine patterns of X-chromosome inactivation and the results were compared to normal females dogs of the same colony. The fourth objective was to

examine type IV collagen gene expression changes in dogs affected with XLHN and ARHN. In doing so, this allowed for the completion of the final objective, the identification of the mutation causative for ARHN in the ECS.

CHAPTER II

DEVELOPMENT OF A GENOMIC TEST FOR X-LINKED HEREDITARY NEPHROPATHY IN A MIXED BREED DOG

Overview

A colony of mixed breed of dogs, termed Navasota or NAV dogs, which has XLHN, was established at Texas A&M University. This colony is an ideal model for the study of human XLAS. When this colony was first established, genotypes were determined by skin immunohistochemistry. After the discovery of the causative mutation in *COL4A5*, RNA was used as the template for the detection of the deletion. However, these tests were not as easily performed, or as accurate as was desired. Therefore, a test based on the genomic DNA sequence of *COL4A5* was developed. The sequence of the amplicon was verified and dogs of known clinical status were genotyped. All resulting genotypes were consistent with clinical status. This test allows the genotype of the NAV dogs to be determined at an early age. The test is 100% accurate and more than 200 dogs have been genotyped.

Introduction

In 1998 Dr. George Lees established a colony of mixed-breed dogs, termed NAV dogs, which present with XLHN (Lees et al., 1999). These dogs proved to be an excellent model for the study of human XLAS because the cause of disease and progression of disease are similar. Both XLHN and XLAS are caused by mutations in the *COL4A5* gene which is located on the human and canine X-chromosomes (Hostikka

et al., 1990; Sugimoto et al., 1994). In the NAV dogs, this disease is caused specifically by a ten base pair deletion in exon nine of *COL4A5* (Cox et al., 2003).

This colony of dogs provides researchers with the ability to study not only XLHN but also to investigate various aspects of canine chronic renal failure and its management. In order to properly plan experiments and for the disease progression to be properly monitored, it is essential to know the genotypes of all dogs at an early age.

Before the causative mutation was identified, the preferred method of diagnosis was immunohistochemistry of skin. This method detected the α5(IV) protein in the epidermal basement membrane (EBM). This required skin biopsies to be taken, which is an invasive procedure for a young dog to endure. In addition, this method of diagnosis had a history of false results, especially for the carrier females.

After the identification of the causative mutation, genetic status was determined by detection of the ten base pair deletion using RNA as the template. RNA was used because, at that time, only the coding sequence of the gene was known. This method was also problematic because RNA had to be isolated from either skin biopsies (again, an invasive procedure) or blood, which is an unreliable source. In addition, work using RNA is, at times, difficult.

With the publication of the canine genome sequence by the NHGRI in 2004 (Lindblad-Toh et al., 2005), the entire sequence of the *COL4A5* gene was made available and a genomic test could be developed that is both accurate and noninvasive. Genomic DNA is more easily obtained than either skin biopsies or RNA and can be easily isolated from blood or buccal swabs. The collection of buccal swabs is ideal because it is

noninvasive and can be performed when the dogs are only a few days old. The use of this test has proven to be fast, economical and 100% accurate.

Materials and methods

DNA was isolated from blood or buccal swabs using the Puregene DNA Purification kit according to the manufacturer's instructions (Gentra Systems, Inc., Minneapolis, MN). DNA was isolated from tissue using the Qiagen DNeasy kit (Qiagen Inc., Valencia, CA). Quantity and quality of DNA were determined by spectrophotometry using a NanoDrop 1000 (NanoDrop Tech., Wilmington, DE).

Primers in the intronic regions flanking exon nine of *COL4A5* were designed based on the published canine genome sequence. These primers were designed to amplify 202 base pairs of sequence including exon nine of *COL4A5*: Forward 5′-CGCTTGACTATTTGTGTGTCATAA-3′, Reverse 5′-AAGGTGATGCTGTGATCTGATTTA-3′. Amplification by PCR was carried out with each 10μl reaction containing 50ng of DNA, 3.0mM of MgCl, 0.5mM of each dNTP, 1.0μM of each primer (forward and reverse), 1X MasterAmp (Epicentre Biotechnologies, Madison, WI, USA), 1X *Taq* DNA Polymerase Buffer B (Fisher Scientific, Pittsburgh, PA, USA) and 0.04 U/μl of *Taq* DNA Polymerase (Fisher Scientific). Cycling conditions were as follows: 95°C for two minutes, followed by five cycles of 95°C for 30 seconds, 58°C for 15 seconds and 72°C for ten seconds, followed by an additional 30 cycles of 95°C for 20 seconds, 56°C for 15 seconds, 72°C for ten seconds and then a single cycle at 72°C for five minutes.

The product sequence was initially verified by purifying seven µl of the PCR product using 10U of Exonuclease I (Epicentre) and 1U of Shrimp Alkaline Phosphatase (Roche, Indianapolis, IN, USA) incubated at 37°C for 30 minutes, followed by a 15 minute incubation at 80°C. The Big Dye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was used, following the manufacturer protocol, for nucleotide sequencing. Sequences were resolved on an ABI3130 (Applied Biosystems). All subsequent genotypes were resolved on an ABI3130 and analyzed using Genotyper or Genemapper software (Applied Biosystems).

Results and discussion

The sequencing results confirmed that the primers amplified exon nine of *COL4A5* and the 59 base pairs and 60 base pairs in introns eight and nine, respectively. The sequence of exon nine of *COL4A5* of normal dogs shows no point mutations, insertion or deletions but affected dogs have the ten base pair deletion in this gene.

To assure that the test was indeed accurate, genotypes from known normal dogs of both genders, XLHN-affected males and XLHN-carrier females were determined in a blind test. XLHN-affected males had a single product at 192 base pairs, normal dogs of both genders had a single product at 202 base pairs and XLHN-carrier females had two products; one at 192 and the other at 202 base pairs. Examples of results are in Figure 2.1. All genotypes were consistent with the known clinical status of the dogs.

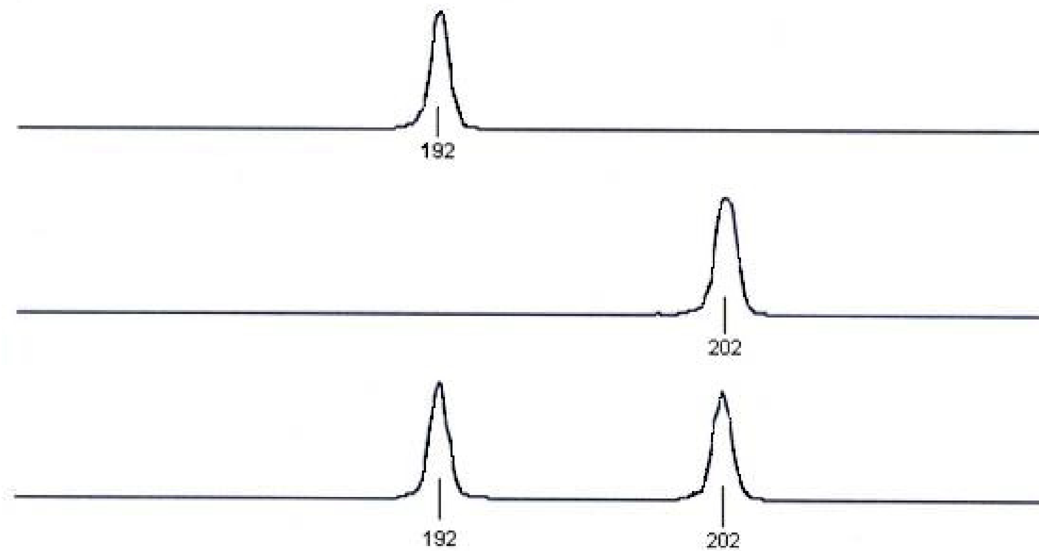

Fig. 2.1. Example of a mutation based test for X-linked hereditary nephropathy in the Navasota dog. Shown are Dog 1 (affected male), Dog 2 (normal male) and Dog 3 (carrier female).

To date over 200 NAV dogs have been genotyped and the accuracy of this test is 100%. Buccal swabs can be collected when dogs are only a few days old and results are available within two weeks of birth. This method allows for better planning for future procedures involving these dogs.

CHAPTER III

X-CHROMOSOME INACTIVATION PATTERNS IN NORMAL AND X-LINKED HEREDITARY NEPHROPATHY CARRIER DOGS

Overview

AS and HN are glomerular nephropathies caused by mutations in the genes encoding the type IV collagens. In a mixed breed of dog, NAV dogs, XLHN is caused by a ten base pair deletion in exon nine of *COL4A5*. Males harboring this mutation succumb to end-stage renal disease before 18 months of age. In contrast, female carriers of this disease survive much longer, most have a normal life-span, and vary in disease progression as compared with XLHN-affected males. X-chromosome inactivation (XCI) patterns have been studied in *human* X-linked AS carriers and some have been shown to have a high degree of skewed XCI. However, similar studies have never been reported in an *animal* model of this disease. Therefore, patterns of XCI were examined in XLHN-carrier NAV dogs. The variation in XCI among the 26 XLHN-carrier and seven normal female NAV dogs studied was low and only three were found to be highly skewed (>80%). The average skewedness among all dogs was 59%. Of these 33 dogs, the 26 XLHN-carriers showed an average skewedness of 57%, and all three of the highly skewed dogs were carriers. When the two groups were compared using Fisher's exact test, no significant difference was found (P=0.477). Given the low variance among the dogs studied and the mean skewedness of 59%, it is clear that the majority of these dogs have random X-chromosome inactivation. In this population, genotype does not seem to have an effect on X-chromosome inactivation. Highly skewed X-chromosome

inactivation also appears to be random, given that no difference was observed between the XLHN-carriers and normal females. Because of the low amount of skewedness seen, these dogs may not be a suitable model in which to study a potential correlation between X-chromosome inactivation and disease progression.

Introduction

AS and HN refer to any of the inherited glomerular nephropathies caused by mutations in any of the three members of the type IV collagen gene family that are required for normal glomerular basement membrane (GBM) structure and function. These conditions occur in both the human (AS) and the domestic dog (HN). Both AS and HN can be inherited in three fashions: X-linked, autosomal recessive and autosomal dominant.

The *COL4A5* gene is located on the X-chromosome in both the human and the dog and mutations in this gene are responsible for the X-linked forms of AS and HN. Unlike the autosomal forms of AS and HN, males and females harboring a *COL4A5* mutation exhibit drastically different phenotypes (Jansen et al., 1987; Hostikka et al., 1990; Lees et al., 1999; Jais et al., 2000; Jais et al., 2003; Lowe et al., 2003). Because they are hemizygous, affected male humans and dogs will have *only* a mutated copy of the *COL4A5* gene and will, on average, progress quickly to ESRD. Alternatively, female carriers of both species are heterozygous (having both a wild type and mutated copy of the gene) and human female carriers exhibit highly variable phenotypes, ranging from mild hematuria to ESRD (Jais et al., 2003).

Currently, there exist two spontaneously occurring canine models of XLHN: 1) a Samoyed kindred and 2) a kindred of a mixed breed dog, termed NAV dogs (Bernards and Valli., 1977; Lees et al., 1999). The Samoyed kindred harbors a nonsense mutation in exon 35 of *COL4A5* and the NAV kindred harbors a ten base pair deletion in exon nine of *COL4A5*, leading to a premature stop codon in exon ten (Zheng et al., 1994; Cox et al., 2003). In addition, an X-linked AS (XLAS) murine model has been engineered to have a nonsense mutation in exon one of *COL4A5* (Rheault et al., 2004).

XLHN-affected males in the NAV kindred tend to progress at similar rates, with the onset of proteinuria at three to four months, and ESRD by six to15 months of age (Lees et al., 1999). XLHN-carrier NAV females exhibit clinical manifestations similar to the XLHN-affected males in that all will develop proteinuria, but the magnitude of proteinuria varies and the rate of disease progression is slower (Lees et al., 1999). Proteinuria is first detected in carriers at an average of four months of age and will increase until approximately 12 months of age, at which point it will remain stable for many years. Most XLHN-carrier NAV dogs have a normal life span, which is substantially longer than their affected male counterparts. This ability to retain stable renal function for many years is a phenomenon that is not well understood.

It has been proposed that the high degree of variation in human female XLAS carriers may be due, in part, to non-random (skewed) X-chromosome inactivation (XCI) patterns, and multiple studies have investigated this phenomenon (Vetrie et al., 1992; Guo et al., 1995; Nakanishi et al., 1998; Shimizu et al., 2006; Kashtan, 2007). Despite their availability, to date, no studies have reported XCI patterns in any of the existing

animal models of X-linked AS and HN. Therefore, this investigation was carried out in order to determine the XCI patterns in normal and XLHN-carrier NAV dogs.

The XCI patterns in seven normal and 26 XLHN-carrier female NAV dogs were studied using an assay similar to that used in human studies. Two variable length CAG repeats found in the first exon of the androgen receptor (*AR*) gene can be used to distinguish the paternal and maternal X-chromosomes (Shibuya et al., 1993; Lu et al., 2001). XCI patterns in the 33 female NAV dogs were determined using a methylation-sensitive PCR assay that analyzes one of these CAG repeats (Allen et al., 1992). In order to decrease the need for tissue samples, an initial study was performed to test the correlation of XCI patterns found in peripheral blood and renal tissue. There was a high degree of correlation between the two sample types and this allowed additional dogs to be profiled using only peripheral blood. Few XLHN-carriers NAV dogs were found to be highly skewed and variation within the population was very low. These findings demonstrate that, in this population, the genotype of females does not seem to influence XCI. The apparent rarity of skewed XCI in this population complicates any future studies to correlate XCI patterns and disease progression. Any phenotypic variation seen in this population is likely due to other contributing genetic factors.

Materials and methods

Genotype determination and sample collection

Buccal swabs collected at birth were used to determine genotype of the NAV dogs by PCR amplification of exon nine of *COL4A5* using the following intronic

primers: Forward 5′-CGCTTGACTATTTTGTGTGTCATAA-3′, Reverse 5′-AAGGTGATGCTGTGATCTGATTTA-3′. Amplification by PCR was carried out with each 10μl reaction containing 50ng of DNA, 3.0mM of MgCl, 0.5mM of each dNTP, 1.0μM of each primer (forward and reverse), 1X MasterAmp (Epicentre Biotechnologies, Madison, WI), 1X *Taq* DNA Polymerase Buffer B (Fisher Scientific, Pittsburgh, PA) and 0.04 U/μl of *Taq* DNA Polymerase (Fisher Scientific). Cycling conditions were as follows: 95°C for two minutes, followed by five cycles of 95°C for 30 seconds, 58°C for 15 seconds and 72°C for ten seconds, followed by an additional 30 cycles of 95°C for 20 seconds, 56°C for 15 seconds, 72°C for ten seconds and then a single cycle at 72°C for five minutes. Genotypes were resolved on an ABI 3130 and analyzed using Genemapper software (Applied Biosystems, Foster City, CA).

For the reported study, peripheral blood was collected from 25 XLHN carrier, seven normal female NAV dogs and one normal male control (as a digestion control) and stored in EDTA tubes. Renal tissue from ten dogs was collected by biopsy or at the time of necropsy. Tissue was either frozen in liquid nitrogen or stored in RNAlater (Ambion Inc., Austin, TX).

DNA isolation

DNA was isolated from blood and buccal swabs using the Puregene DNA Purification kit, and the manufacturers instructions were followed (Gentra Systems, Inc., Minneapolis, MN). DNA was isolated from tissue using the Qiagen DNeasy kit (Qiagen

Inc., Valencia, CA). Quantity and quality of DNA were determined by specrophotometry using a NanoDrop 1000 (NanoDrop Tech., Wilmington, DE).

Sequencing of a portion of the canine androgen receptor gene

Primers used to amplify the first and second CAG repeats in the canine *AR* gene are as follows: Forward 5′- CGAAGTGATCCAGAACCCGG-3′, Reverse 5′- GCTACCTGGCTCTGGATGAGGAA-3′ and Forward 5′- CCCATCCACATTGTCACTGCTG-3′, Reverse 5’- CATGGACACCGACACTGCCTT-3′, respectively. Each reaction contained 50ng of DNA, 3.0mM of MgCl, 0.5mM of each dNTP, 1.0μM of each primer (forward and reverse), 1X MasterAmp (Epicentre Biotechnologies), 1X *Taq* DNA Polymerase Buffer B (Fisher Scientific) and 0.04 U/μl of *Taq* DNA Polymerase (Fisher Scientific). Reaction conditions were as follows: 95°C for five minutes, followed by 33 cycles of 95°C for 30 seconds, 60°C for 15 seconds and 72°C for ten seconds and a single cycle at 72°C for five minutes. Seven μl of the PCR product was purified using 10U of Exonuclease I (Epicentre) and 1U of Shrimp Alkaline Phosphatase (Roche, Indianapolis, IN) and incubated at 37°C for 30 minutes, followed by a 15 minute incubation at 80°C. The Big Dye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems) was used, following the manufacturer protocol, for nucleotide sequencing, sequences were resolved on an ABI3130 (Applied Biosystems).

X-chromosome inactivation analysis

Two hundred ng of DNA was combined with 1U each of the methylation-sensitive restriction enzymes *Hpa*II and *Hha*I in a 50μl reaction with 1X Buffer 4 and 100 μg/ml Bovine Serum Albumin (New England Biolabs, Inc., Ipswich, MA). In addition, undigested controls of each sample were incubated in 50μl reactions without the restriction enzymes. This served as a control for any effects the restriction enzyme buffers may have had on subsequent PCRs. All reactions (digested and undigested) were incubated for 16 hours at 37°C followed by inactivation for 20 min at 65°C.

From each of the digested and undigested DNA samples 25ng was used for the subsequent PCR. All dogs were analyzed for either the first or second CAG repeat in the *AR* gene using the primers and conditions described in the previous section. All reactions were run in triplicate to ensure reproducible and accurate results.

Genotypes were resolved on an ABI 3130 and analyzed using Genemapper software (Applied Biosystems). Degree of skewedness was determined by comparing the peak areas of the digested and undigested samples. The following calculation was used: skewing= (Bd1/Bu1)/[(Bd1/Bu1)+(Bd2/Bu2)] where B1 and B2 represent the first and second alleles, respectively, and Bd and Bu represent the digested and undigested samples, respectively (Lau et al., 1997).

Statistical analysis

A paired Student's t-test was used to compare the inactivation patterns seen in blood and tissue (α=0.01). Fisher's exact test was used to compare the occurrence of highly skewed individuals in the XLHN-carriers and normal females.

Results and discussion

X-chromosome inactivation

The fact that females have two X-chromosomes gives them an obvious advantage in X-linked diseases. Unlike males, who have only one X-chromosome, a female must carry a mutation on both X-chromosomes to be fully affected by an X-linked disease, and this is a rare occurrence. Typically, males will be fully affected and carrier females will show a range of symptoms between normal and affected.

It has been hypothesized that this range of symptoms may be due to unequal, or skewed, XCI (Vetrie et al., 1992). In a female, each cell must inactivate one X-chromosome as a mechanism of dosage compensation (Lyon, 1961). As this is thought to be a random process, most females will have a 50:50 ratio of maternal to paternal XCI. However, there are documented cases of unequal, or skewed, XCI (Guo et al., 1995; Lau et al., 1997; Uz et al., 2007). Skewing favors the inactivation of one X-chromosome over the other, and when this occurs in carriers of an X-linked disease it can have an effect on the clinical presentation. If the X-chromosome harboring a mutation is more often active, the phenotype may be more severe because less of the

normal protein is produced. In contrast, if it is the wild-type X-chromosome that is more often active, the phenotype may be more like that of a normal female.

Reports of human carriers of XLAS have shown that a wide range of phenotypes exist. An extensive study by Jais et al. found carriers developing ESRD as early as age 19, while some retained adequate renal function into and beyond the sixth decade of life (Jais et al., 2003). Some human XLAS-carriers with early ESRD have been shown to have skewed XCI and it has been proposed that differences in XCI may account for some differences in phenotype (Vetrie et al., 1992; Guo et al., 1995). Similar studies involving any of the previously described animal models of AS or HN have not been reported. Thus, this study was done to investigate the patterns of XCI in XLHN-carrier and normal female NAV dogs.

Analysis of X-chromosome inactivation

To estimate XCI patterns, a methylation sensitive PCR that amplifies one of the CAG repeats in the first exon of the AR gene was used. This assay has been successfully used for studies of human diseases since 1992 (Allen et al., 1992). Differences in the lengths of the two repeats in the first exon of the canine *AR* gene, and the one found in the human, can be used to distinguish the paternal and maternal X-chromosomes (Shibuya et al., 1993; Lu et al., 2001). The DNA analyzed using this method of analysis was first digested by two methylation-sensitive restriction enzymes: *Hpa*II and *Hha*I. Due to the mechanism of XCI, the inactive X-chromosome and the *AR* gene located on it will be methylated, preventing digestion by *Hpa*II and *Hha*I. In contrast, the DNA on the

active X-chromosome is unmethylated and is easily digested. The DNA from the initial digest was then used in a PCR that amplifies the aforementioned CAG repeat. Because it was protected from digestion, only the DNA located on the *inactive* X-chromosome will be amplified. The degree of skewedness is expressed as a ratio of the products amplified from the two alleles. An example of highly skewed and unskewed individuals can be seen in figure 3.1.

DNA from a male was used as a standard control for this assay because the single X-chromosome will be active and unmethylated. That is, when the digestions work properly, there cannot be any amplification because the DNA encoding the *AR* gene has been completely digested. This was found to be the case in our control (Fig. 3.1).

Prior to their inclusion in the study, over 50 dogs were genotyped for the CAG repeats because only those that were heterozygous for at least one of these alleles would be informative. Due to the breeding structure of this colony a majority of dogs were homozygous and therefore excluded, reducing the sample size. In addition, the second repeat was found to be less informative than the first, so the majority of dogs were assayed using the first CAG repeat. The recent introduction of a new sire increased the heterogeneity of this population, and the majority of dogs used in this study were his offspring.

Comparison of blood and tissue samples

Because HN is a renal disease, any XCI patterns studied should ideally be performed using renal tissue. However, most studies have used peripheral blood,

although some have used skin biopsies (Vetrie et al., 1992; Guo et al., 1995; Nakanishi et al., 1998; Shimizu et al., 2006; Wang et al., 2007). One study provided evidence that

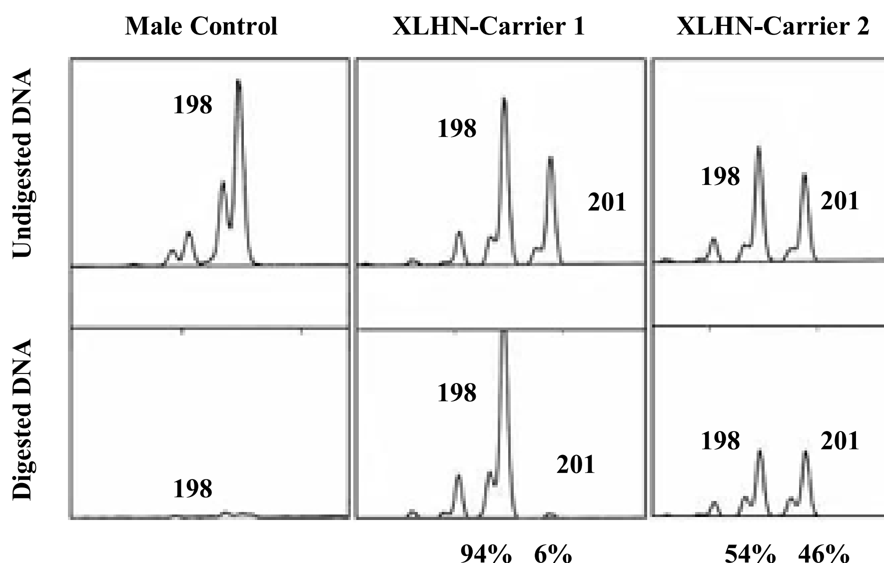

Fig. 3.1. Genotypes from a normal male control, and skewed and unskewed XLHN-carrier NAV dogs.Genotypes from digested and undigested DNA samples are shown. No product is seen for the digested male control because HpaII and HhaI digestion of the X-chromosome was complete. The genotypes from the two undigested XLHN-carrier female DNA samples show two alleles (198 and 201). The genotypes from the two digested XLHN-carrier female DNA samples differ because of different patterns of XCI. XLHN-carrier #1 has highly skewed XCI, and therefore the peak heights of the two alleles are strikingly different. XLHN-carrier #2 has equal XCI, both alleles amplify equally well after digestion.

XCI patterns in blood accurately estimate XCI patterns in renal tissue, but this was performed with only one sample and therefore needs further verification (Guo et al., 1995). Sharp et al. conducted an extensive study in normal women, which showed that, in spite of a few outliers, there was an overall significant correlation between blood,

buccal swab and urinary epithelial samples (Sharp et al., 2000). In a more recent study Uz et al. reported similar patterns of XCI in blood and buccal swab samples of seven patients with pre-eclampsia (Uz et al., 2007). In order to verify previous findings and reduce the need for renal tissue (because obtaining it is an invasive procedure), samples of blood and tissue from eight XLHN-carrier NAV dogs were compared.

XCI patterns in blood and tissue samples were compared using a Student's t-test. No statistical difference was found for all but two pairs of samples (P=0.0210, 0.0320, 0.0204, 0.3054, 0.6227, 0.6726; α=0.01). Although the P values obtained from the remaining pairs indicate that there is a difference between the skewedness in blood and tissue (P=0.0002 and P=0.0059), neither sample type showed a high degree of skewedness. That is, the skewedness patterns seen in the first pair (P=0.0059) were 60% and 51% for blood and tissue, respectively. For the second pair (P=0.0002) the skewedness was 44% and 65% for the blood and tissue samples, respectively. Even with the presence of two outliers, these data seems to support the previously reported correlation in XCI patterns between blood and tissue.

Based on these finding and those previously reported, blood from 24 additional female NAV dogs was used in the remainder of the study. Although renal tissue remains the ideal source of DNA for this type of study, these data support previous reports and allowed this study to use peripheral blood in place of renal tissue.

X-inactivation patterns in normal and XLHN-carrier NAV dogs

XCI patterns were studied in a total of 33 female NAV dogs; 26 XLHN-carriers and seven normals. Blood was available from all but two dogs (as they were already deceased); however, tissues from these two dogs were available for analysis. Skewedness patterns ranged from 26% to 94% inactivation of the X-chromosome with the wild-type *COL4A5* (Fig. 3.2). The average skewedness among all dogs was 59%, indicating random and equal XCI. The variance and standard deviation among the 33 dogs were low, 0.026 and 0.161, respectively. The low variance and standard deviation demonstrate that the sample size was sufficient.

Within the group of 26 XLHN-carriers, the variance and standard deviation were 0.029 and 0.172, respectively. The mean skewedness was 57%, again showing that, on average, there was completely random and equal XCI. Among all dogs, only three were found to be highly skewed (>80%) and all were XLHN-carriers. These carriers were 81%, 85% and 94% skewed towards inactivation of the X-chromosome with the wild-type *COL4A5*. It is reasonable to conclude that these dogs would express significantly more of the mutated α5(IV) protein than the wild-type. The low number of highly skewed individuals (9% of the total sample population) suggests that skewed XCI is a rare occurrence.

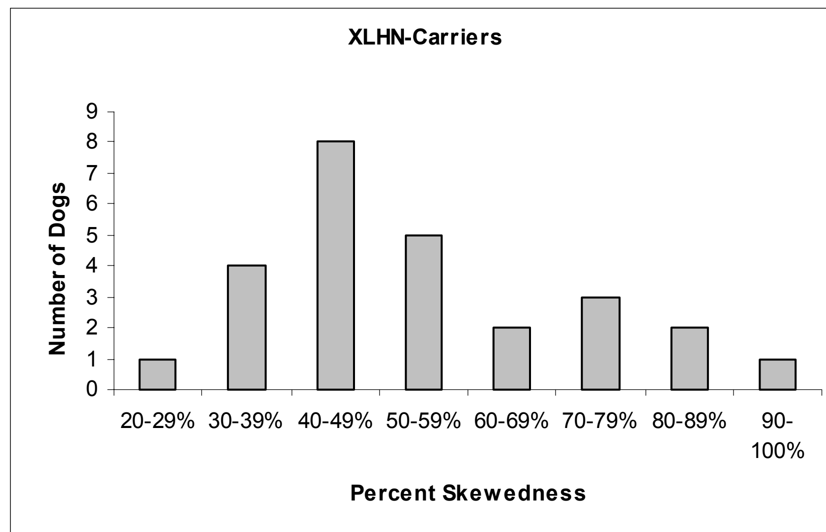

Fig. 3.2. Number of XLHN-carrier females in groups by percent skewedness. This graph shows the distribution of carriers as percent skewedness increases. The majority of dogs are found between 40% and 70% skewedness. This graph demonstrates that most XLHN-carriers are not skewed; most show a random pattern of XCI.

The use of Fisher's exact test to compare the XCI patterns in XLHN-carrier and normal dogs showed that there was no difference between the two groups (P=0.47654). Even when the threshold for skewed inactivation was lowered to 70%, the P value remained high (0.10313). It has been proposed that carriers of X-linked diseases may more often exhibit skewed XCI as a response to the mutation they carry on the X-chromosome, but the majority of studies show that this is a random event (Orstavik, 2006). These data confirm these previous findings; no correlation between clinical status and skewed XCI was observed in female NAV dogs.

X-chromosome inactivation and age

The correlation between age and skewed XCI has been shown to be high in some cases (Kristiansen et al., 2003; Hatakeyama et al., 2004) . In theory, as a woman ages, her degree of skewedness may become more pronounced due to selective pressures on certain cell populations, and higher degrees of skewed XCI have been shown in women of advanced age (Kristiansen et al., 2003) . However, a longitudinal study reported in 2004 found no difference in XCI in blood samples taken 20 years apart (Sandovici et al., 2004). For the current study, ages of the dogs at the time of sample collection ranged from two to 114 months of age, with an average age of 29 months. Among the three XLHN-carriers shown to have highly skewed XCI, one was seven month of age and two were 23 months of age. Therefore, in this population those dogs of older age (>60 months) did not display a higher rate of skewed XCI (Fig. 3.3).

This study is the first to report the use an animal model to investigate XCI patterns in carriers of XLHN or XLAS. These results provide further evidence that peripheral blood accurately estimates the degree of skewedness found in the kidney of XHLN-carriers. The use of blood in future XLHN and XLAS XCI analyses will allow for long term studies to be conducted. In addition, the data have shown that with the exception of three dogs, very similar patterns of XCI exist in this sample population. Therefore, the presence of the *COL4A5* mutation in these carriers does not seem to induce a skewed pattern of XCI. Although additional studies to correlate disease progression and XCI patterns could be performed, the apparent rarity of highly skewed individuals makes conducting such studies impractical.

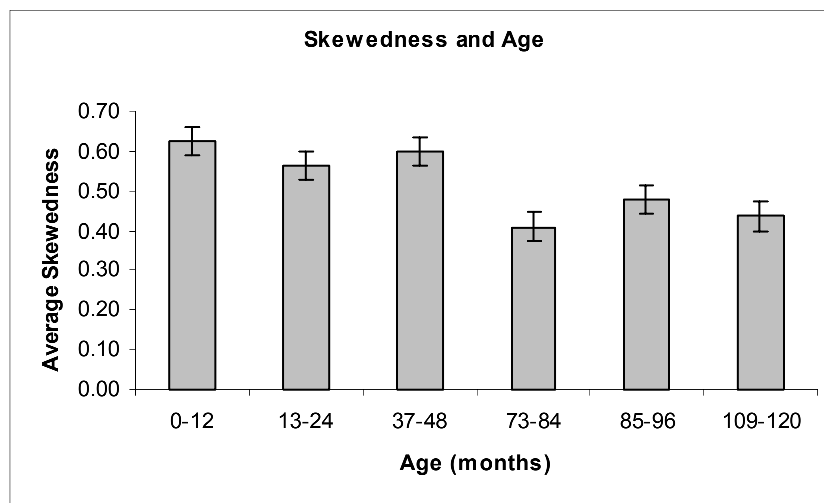

Fig. 3.3. Percent skewedness based on age. Average percentage of XCI is plotted in relation to age. As age increases, there is not a corresponding increase in XCI.

CHAPTER IV

DESIGN OF A SYNTHETIC *COL4A5* GENE FOR GENE THERAPY IN X-LINKED HEREDITARY NEPHROPATHY DOGS

Overview

AS and HN are hereditary glomerular nephropathies in the human and the dog, respectively. These diseases are caused by mutations in the type IV collagens and are transmitted most often in an X-linked fashion, due to mutations in the *COL4A5* gene. Affected patients develop hematuria, proteinuria and eventually ESRD. There are no cures for these diseases; the only treatment options for humans are dialysis and renal transplantation. Gene therapy, however, has been proposed as a treatment for AS and HN. A colony of mixed breed dogs, NAV dogs, which present with XLHN, was established at Texas A&M University. This colony has the potential to be used in gene therapy trials for treatment of XLHN. To this end, a *COL4A5* cDNA sequence was modified and synthesized with the goal of introducing it into the kidneys of affected dogs. The cDNA sequence was modified in order to facilitate its cloning into various vectors and also to distinguish the protein product from native α5(IV). By introducing this wild-type gene, it is possible that the resulting protein will be deposited into and aid in the repair of the GBM.

Introduction

AS and HN are hereditary glomerular diseases that result in ESRD. Although HN is uncommon in the dog and AS accounts for only approximately 3% of all ESRD in

humans, study of the disease process offers the potential to increase our understanding of ESRD (Agodoa et al., 1996). The only therapies currently available for people with AS are dialysis and renal transplantation. However, these options are expensive, the waiting lists for transplants are long and many people never receive the transplant they need to survive. Dialysis and transplantation are currently not feasible options for dogs with HN, but other medical treatments to slow disease progression are similar.

Gene therapy has been proposed as a potential treatment for XLAS and XLHN because these diseases are caused by mutations in a single gene (*COL4A5*). Previous studies demonstrated that the human *COL4A5* cDNA could be introduced into porcine kidneys by perfusion with subsequent α5(IV) deposition in the GBM (Heikkila et al., 2001). This work provided encouraging results for future gene therapy experiments. However, in order to reduce the possibility of immune reactions, the coding sequence used should be from the same species, *i.e.* in a canine model, the canine *COL4A5* sequence should be used. In addition, experiments need to be performed that show that this form of therapy can slow disease progression. Thus, we are trying similar treatments in the NAV dog model of XLHN using a canine-specific cDNA. The ultimate goal is for the wild-type *COL4A5* cDNA to be expressed, and for the encoded protein to subsequently be deposited into the GBM of affected dogs. To this end, we have designed and synthesized a *COL4A5* cDNA.

Materials and methods

Design and synthesis of COL4A5 cDNA

Modifications made to the *COL4A5* cDNA sequence were as follows: 1) a FLAG epitope was inserted into the fifth collagenous interruption and 2) unique restriction sites were added to the 5′ and 3′ ends of the sequence (Fig. 4.1). The resulting sequence was checked using BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/) to assure that the reading frame and amino acid sequence were maintained. Once the nucleotide sequence was modified, it was sent to Blue Heron Biotechnologies (http://www.blueheronbio.com/) (Blue Heron Biotechnologies, Bothell, WA) for synthesis. The cDNA was cloned into the Blue Heron Bio pUCminus vector and sequenced verified by Blue Heron Biotechnologies.

Transfer of COL4A5 into pIRES-hrGFP-2a

In order to transfer the *COL4A5* cDNA into a mammalian expression vector, it was removed from the Blue Heron pUC-minus vector using a double digested with *Not*I and *Sal*I (Promega Corporation, Madison, WI) in a 20µl reaction with final concentrations of 1X Buffer D, 0.2µg Bovine Serum Albumin and 5U of each enzyme. The plasmids were digested at 37˚C for one hour followed by a 65˚ incubation for 15 minutes and the products resolved on an agarose gel. The mammalian expression vector, pIRES-hrGFP-2a, was similarly digested. The digestion products were resolved on a 1% agarose gel, the bands corresponding to the *COL4A5* insert and the pIRES-hrGFP-2a (Stratagene, La Jolla, CA) linear plasmid were cut out and gel extracted using the

QIAquick Gel Extraction kit (Qiagen, Valencia, CA). The *COL4A5* and linear pIRES-hrGFP-2a gel extraction products were ligated together following the NEB Quick Ligase manufacturer's protocol (New England Biolabs, Ipswich, MA) and incubated at room temperature for one hour. Two microliters of each ligations was transformed into SURE® cells (Stratagene).

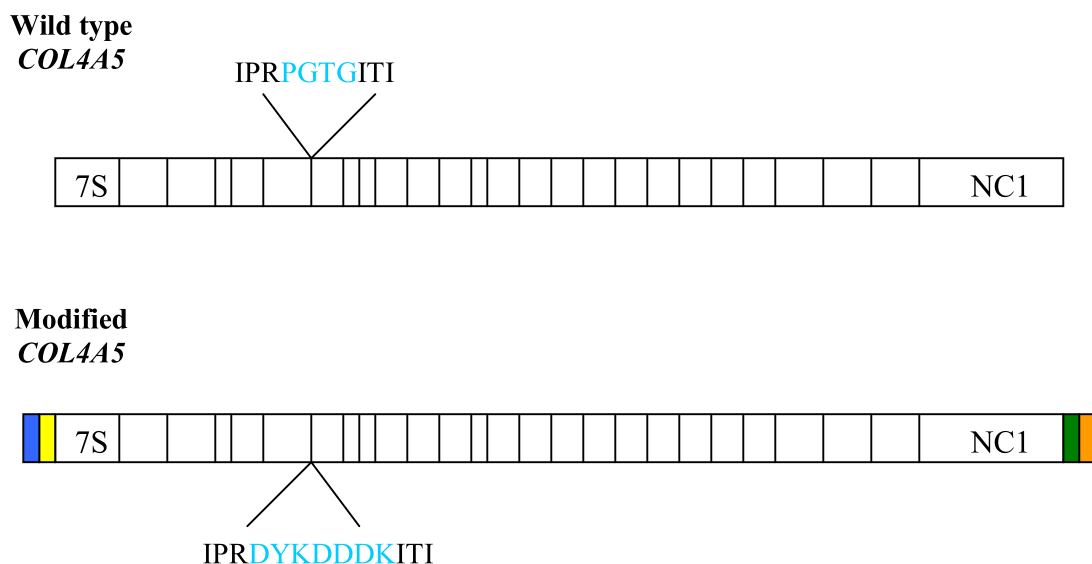

Fig. 4.1. Wild-type and modified *COL4A5* genes. The vertical lines represent interruptions in the collagenous domain of the coding sequence. The modified *COL4A5* gene has a FLAG amino acid sequence inserted into the fifth interruption. In addition, it has four unique restriction sites (two on the 5′ and two on the 3′ ends): *Not*I site in blue, *Sac*II site in yellow, *Sph*I site in green and *Sal*I site in orange.

The transformants were grown on LB plates containing 0.05g/L of Ampicillin. Colonies were picked and cultured overnight and plasmids isolated using the Eppendorf Plasmid Mini Kit (Eppendorf, Westbury, NY). Single digests of the plasmids were

performed with *Not*I and *Sal*I in 20µl reactions with final concentrations of 1X Buffer D, 0.2µg Bovine Serum Albumin and 5U of enzyme (Promega). The plasmids were digested at 37˚C for one hour followed by a 65˚ incubation for 15 minutes and the products resolved on a 1.2% agarose gel. Double digest with both *Not*I and *Sal*I were performed as previously described (Promega). The products were run on a 1.2% agarose gel along with undigested plasmids. The resulting plasmids that were suspected to have the *COL4A5* insert were subjected to a second set of single digests using *Bgl*II and *Hind*III in 20µl reactions with final concentrations of 1X Buffer B, 0.2µg Bovine Serum Albumin and 5U of enzyme (Promega Corp.). Double digests were performed using both *Bgl*II and *Hind*III in 20µl reactions with 1X Buffer B, 0.2µg Bovine Serum Albumin and 5U of each enzyme (Promega Corp.). Products were resolved on 1.2% agarose gels.

Transfer of COL4A5 into pcDNA3.1/CT-GFP-TOPO

The *COL4A5* cDNA will be transferred into the pcDNA3.1/CT-GFP-TOPO mammalian expression vector (Invitrogen, Carlsbad, CA). The entire *COL4A5* insert was amplified from the Blue Heron pUCminus vector using the following primers: Forward 5'-CATGGCGGCCGCGCGGCACAAACTCAAGATT-3', Reverse 5'-GAGAGCGGCCGCGGCTACAGTCAGTGGAGAGGA-3'. Amplification was performed using the Advantage GC Genomic LA Polymerase kit (Clontech, Mountain View, CA) following the manufacturer's protocol with 100ng of plasmid DNA. Reactions conditions are as follows: 95˚ for five minutes, 56˚ for 30 seconds, 72˚ for six minutes followed by 35 cycles of 95˚ for one minute, 71˚ for 30 seconds and 72˚ for six

minutes. The products were resolved on a 1% agarose gel and gel extracted using the Qiagen Qiaex II Gel Extraction Kit (Qiagen Inc.). Thirty ng of the gel extraction products were then ligated into pcDNA3.1/CT-GFP-TOPO following the manufacturer's protocol and incubated at room temperature for one hour. Two µl of the ligation mixture was then transformed into *Escherichia coli* TOP10 cells (Invitrogen). Additionally, ligations into pcDNA3.1/CT-GFP-TOPO were transformed into *E. coli* EC100 chemically competent cells (Epicentre) following the suggested protocol. Plasmids were isolated from the resulting colonies using the laboratory STE protocol. The plasmids were screened for the *COL4A5* insert by PCR amplification using the above described primers and conditions.

Plasmids for which positive amplification of *COL4A5* was detected were sequenced using the Big Dye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using following protocol: 3µl plasmid, 2µl of Big Dye v. 1.1 and 1µl of the appropriate primer. Two of the primers (T7 forward and GFP reverse) used for sequencing were provided by Invitrogen, the third was designed to capture the internal portion of the *COL4A5* cDNA: Forward 5'-GTCATCACTGCCAGGACC-3'. The sequencing reaction used was as follows: 96˚ for two mintues followed by 30 cycles of 96˚ for 30 seconds, 55˚ for 15 seconds and 60˚ for four minutes.

Transfections

HEK293 cells were maintained using E-MEM media from ATCC (ATCC, Manassas, VA) supplemented with 10% FBS (Invitrogen), 100U of Penicillin and 100ug

of Streptomycin (Invitrogen). 500,000 cells were pre-plated in each well of a six well plate and allowed to adhere and grow for 24 hours prior to transfection. Cells were transiently transfected using 7.5µl Lipofectamine 2000 (Invitrogen) in each well of a six well plate. Plasmid DNA concentrations varied from 6.0µg of the control vector (pIRES-hrGPF-2a) to 8.0µg of the pIRES-hrGFP-2a with *COL4A5* insert. The media was replaced five to six hours post-transfection. Transfection efficiency was estimated by visual analysis of GFP expression 24 and 48 hours post-transfection.

Results and discussion

The synthesized *COL4A5* cDNA was confirmed by sequencing performed by Blue Heron Biotechnologies. The cDNA can be transferred into various vectors using restriction digestion that assures directional cloning. This method was used to transfer the *COL4A5* cDNA into pIRES-hrGFP-2a. Verification of transfer into this vector was performed by restriction digestion and all resulting products were of the expected sizes.

The FLAG sequence inserted into the fifth collagenous interruption will be used to distinguish the introduced α5(IV) from native α5(IV). Although XLHN-affected male NAV dogs will not produce α5(IV), these dogs will not be used for initial transfer experiments. Initially, *in vitro* expression studies in HEK293 cells will be performed, and the α5(IV) protein will be detected by western blotting with a FLAG antibody. Once we have verified that the cDNA does encode a α5(IV) protein of the correct size, *in vivo* efforts to transfer the cDNA into the kidneys of XLHN-carrier female NAV dogs will be undertaken. The carrier females have both a wild-type and mutated copy of *COL4A5* and

thus, the introduced α5(IV) will need to be distinguished from the wild-type protein. The insertion of this epitope did not alter the reading frame. Figure 4.2 shows the alignment of the amino acid sequences, they are identical except for the FLAG epitope.

```
COLW   MKLRGVSLAAGWFLLALSLWGQPAEAAACYGCSPGSKCDCSGVKGEKGERGFPGLEGHPG 60
COLM   MKLRGVSLAAGWFLLALSLWGQPAEAAACYGCSPGSKCDCSGVKGEKGERGFPGLEGHPG 60
       ************************************************************

COLW   LPGFPGPEGPPGPRGQKGDDGIRGPPGPKGIRGPPGLPGFPGTPGLPGMPGHDGAPGPQG 120
COLM   LPGFPGPEGPPGPRGQKGDDGIRGPPGPKGIRGPPGLPGFPGTPGLPGMPGHDGAPGPQG 120
       ************************************************************

COLW   IPGCNGTKGERGFPGSPGFPGLEGPPGPPGIPGMKGEPGSIIMSSLPGPKGNPGYPGPPG 180
COLM   IPGCNGTKGERGFPGSPGFPGLEGPPGPPGIPGMKGEPGSIIMSSLPGPKGNPGYPGPPG 180
       ************************************************************

COLW   IQGPAGPTGLPGPIGPPGPPGLMGPPGPPGLPGPKGNMGLNFQGPKGEKGEQGLQGPPGP 240
COLM   IQGPAGPTGLPGPIGPPGPPGLMGPPGPPGLPGPKGNMGLNFQGPKGEKGEQGLQGPPGP 240
       ************************************************************

COLW   PGQISEQKRPIDVEFQKGDQGLPGDRGPPGPPGIRGPPGPPGGMKGEKGEQGEPGKRGKP 300
COLM   PGQISEQKRPIDVEFQKGDQGLPGDRGPPGPPGIRGPPGPPGGMKGEKGEQGEPGKRGKP 300
       ************************************************************

COLW   GKDGENGQPGIPGLPGDPGYPGEPGRDGEKGQKGDIGSTGPPGLVIPRPGTG----VTVG 356
COLM   GKDGENGQPGIPGLPGDPGYPGEPGRDGEKGQKGDIGSTGPPGLVIPRDYKDDDDKVTVG 360
       ************************************************        ****

COLW   EKGNMGLPGLPGEKGERGFPGIQGPPGLPGPPGTAVMGPPGPPGFPGERGQKGDEGPPGI 416
COLM   EKGNMGLPGLPGEKGERGFPGIQGPPGLPGPPGTAVMGPPGPPGFPGERGQKGDEGPPGI 420
       ************************************************************

COLW   SIPGFPGLDGQPGAPGLRGPPGPPGPHISPSDEICETGPPGPPGSPGDRGLQGEQGVKGD 476
COLM   SIPGFPGLDGQPGAPGLRGPPGPPGPHISPSDEICETGPPGPPGSPGDRGLQGEQGVKGD 480
       ************************************************************

COLW   KGDTCFNCIGTGVSGPRGQPGLPGLPGPPGSLGFPGQKGEKGHAGLTGPKGLTGIPGAPG 536
COLM   KGDTCFNCIGTGVSGPRGQPGLPGLPGPPGSLGFPGQKGEKGHAGLTGPKGLTGIPGAPG 540
       ************************************************************

COLW   PPGFPGSKGEPGDVLTFPGMKGDKGELGYPGAPGLPGLPGTPGQDGLPGLPGPKGEPGGI 596
COLM   PPGFPGSKGEPGDVLTFPGMKGDKGELGYPGAPGLPGLPGTPGQDGLPGLPGPKGEPGGI 600
       ************************************************************
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Fig. 4.2. Continued

```
COLW  AFKGERGPPGNPGLPGLPGNRGPMGPVGFGPPGPVGEKGIQGVAGNPGQPGIPGPKGDPG 656
COLM  AFKGERGPPGNPGLPGLPGNRGPMGPVGFGPPGPVGEKGIQGVAGNPGQPGIPGPKGDPG 660
      ************************************************************

COLW  QTITQPGKPGLPGNPGRHGEVGLPGDPGLPGPPGLPGIPGNKGEPGIPGIGLPGPPGPKG 716
COLM  QTITQPGKPGLPGNPGRHGEVGLPGDPGLPGPPGLPGIPGNKGEPGIPGIGLPGPPGPKG 720
      ************************************************************

COLW  FPGIQGPPGAPGTPGRIGLEGPSGPPGFPGPKGEPGLGLPGPPGPPGLPGFKGTLGPKGD 776
COLM  FPGIQGPPGAPGTPGRIGLEGPSGPPGFPGPKGEPGLGLPGPPGPPGLPGFKGTLGPKGD 780
      ************************************************************

COLW  RGFPGPPGLPGRTGLDGLPGPKGDVGPKGQPGPMGPPGLPGIGVQGPPGPPGIPGPVGEP 836
COLM  RGFPGPPGLPGRTGLDGLPGPKGDVGPKGQPGPMGPPGLPGIGVQGPPGPPGIPGPVGEP 840
      ************************************************************

COLW  GLHGIPGEKGDPGPPGFDVLGPPGERGSPGIPGAPGPMGPPGTPGLPGKAGASGFPGAKG 896
COLM  GLHGIPGEKGDPGPPGFDVLGPPGERGSPGIPGAPGPMGPPGTPGLPGKAGASGFPGAKG 900
      ************************************************************

COLW  EMGMMGPPGPPGPLGIPGRSGVPGLKGDNGLQGQPGPPGPEGEKGGKGEPGLPGPPGPVD 956
COLM  EMGMMGPPGPPGPLGIPGRSGVPGLKGDNGLQGQPGPPGPEGEKGGKGEPGLPGPPGPVD 960
      ************************************************************

COLW  PDLLGSKGEKGDPGLPGIPGVSGPKGYQGLPGDPGQPGLSGQPGLPGPSGPKGNPGLPGK 1016
COLM  PDLLGSKGEKGDPGLPGIPGVSGPKGYQGLPGDPGQPGLSGQPGLPGPSGPKGNPGLPGK 1020
      ************************************************************

COLW  PGLTGPPGLKGSIGDMGFPGPQGVKGSPGPPGVPGQPGSPGLPGQKGEKGDPGVSGIGLP 1076
COLM  PGLTGPPGLKGSIGDMGFPGPQGVKGSPGPPGVPGQPGSPGLPGQKGEKGDPGVSGIGLP 1080
      ************************************************************

COLW  GLPGPKGEAGLPGYPGNPGIKGSMGDTGLPGLPGTPGAKGQPGLPGFPGTPGLPGPKGIN 1136
COLM  GLPGPKGEAGLPGYPGNPGIKGSMGDTGLPGLPGTPGAKGQPGLPGFPGTPGLPGPKGIN 1140
      ************************************************************

COLW  GPPGNPGLPGEPGPVGGGGRPGPPGPPGEKGNPGQDGIPGPAGQKGEPGQPGFGIPGPPG 1196
COLM  GPPGNPGLPGEPGPVGGGGRPGPPGPPGEKGNPGQDGIPGPAGQKGEPGQPGFGIPGPPG 1200
      ************************************************************

COLW  LPGLSGQKGDGGLPGIPGNPGLPGPKGEPGFQGFPGVQGPPGPPGSPGPALEGPKGNPGP 1256
COLM  LPGLSGQKGDGGLPGIPGNPGLPGPKGEPGFQGFPGVQGPPGPPGSPGPALEGPKGNPGP 1260
      ************************************************************

COLW  QGPPGRPGPTGFQGLPGPEGPRGLPGNGGIKGERGNPGQPGQPGLPGLKGDQGPPGIQGN 1316
COLM  QGPPGRPGPTGFQGLPGPEGPRGLPGNGGIKGERGNPGQPGQPGLPGLKGDQGPPGIQGN 1320
      ************************************************************

COLW  PGRPGLNGMKGDPGLPGVPGFPGMKGPSGVPGSAGPEGDPGLVGPPGPPGLPGPSGQSII 1376
COLM  PGRPGLNGMKGDPGLPGVPGFPGMKGPSGVPGSAGPEGDPGLVGPPGPPGLPGPSGQSII 1380
      ************************************************************
```

Fig. 4.2. Continued

```
COLW  IKGDVGPPGIPGQPGLKGLPGLPGPQGLPGPIGPPGDPGRNGLPGFDGAGGRKGDPGLPG 1436
COLM  IKGDVGPPGIPGQPGLKGLPGLPGPQGLPGPIGPPGDPGRNGLPGFDGAGGRKGDPGLPG 1440
      ************************************************************

COLW  QPGTRGLDGPPGPDGMQGPPGPPGTSSIAHGFLITRHSQTTDAPQCPHGTVQIYEGFSLL 1496
COLM  QPGTRGLDGPPGPDGMQGPPGPPGTSSIAHGFLITRHSQTTDAPQCPHGTVQIYEGFSLL 1500
      ************************************************************

COLW  YVQGNKRAHGQDLGTAGSCLRRFSTMPFMFCNINNVCNFASRNDYSYWLSTPEPMPMSME 1556
COLM  YVQGNKRAHGQDLGTAGSCLRRFSTMPFMFCNINNVCNFASRNDYSYWLSTPEPMPMSME 1560
      ************************************************************

COLW  PLKGQSIQPFISRCAVCEAPAVVIAVHSQTIQIPHCPHGWDSLWIGYSFMMHTSAGAEGS 1616
COLM  PLKGQSIQPFISRCAVCEAPAVVIAVHSQTIQIPHCPHGWDSLWIGYSFMMHTSAGAEGS 1620
      ************************************************************

COLW  GQALASPGSCLEEFRSAPFIECHGRGTCNYYANSYSFWLATVDVSDMFSKPQSETLKAGD 1676
COLM  GQALASPGSCLEEFRSAPFIECHGRGTCNYYANSYSFWLATVDVSDMFSKPQSETLKAGD 1680
      ************************************************************

COLW  LRTRISRCQVCMKRT 1691
COLM  LRTRISRCQVCMKRT 1695
      ***************
```

Fig. 4.2. Comparison of the wild-type (COLW) and modified (COLM) *COL4A5* amino acid sequences. The FLAG epitope is highlighted in yellow.

Unfortunately, once the cDNA was transferred into the pIRES-hrGFP-2a mammalian expression vector and used for transfection into HEK293 cells, no GFP was detected (efficiency was <1%). The absence of detectable GFP indicates a failure to express the α5(IV) protein. Further transfection experiments demonstrated that even the empty pIRES-hrGPF-2a vector was not capable of driving expression of the GFP marker; transfection efficiency was <1%. The fact that the empty vector was not sufficiently strong enough to produce GFP led to a search for an alternate vector.

The Invitrogen pcDNA3.1/CT-GFP-TOPO vector was chosen because of its previously described use in the *in vitro* expression of a canine *COL4A5* cDNA (Harvey

et al., 2003). Efforts to insert the synthetic *COL4A5* cDNA into this vector have not produced the desired results. Initial attempts were made using the TOP10 cells provided by Invitrogen. These cells, however, do not seem to be sufficiently inert with respect to genetic recombination. That is, all of the *COL4A5* inserts have been significantly shorter than expected indicating significant rearrangement. When the inserts were amplified, product sizes were only 1.1Kb, opposed to the expected full length 5.3Kb. Sequencing of the inserts confirmed the presence of only the first 1080 bp of the *COL4A5* cDNA (Fig. 4.3)

```
COL   -------------CACAAACTCAAGATTATGAAACTGCGTGGAGTCAGCCTGGCTGCCGG 47
A     GGCGGCCGCGCGGCACAAACTCAAGATTATGAAACTGCGTGGAGTCAGCCTGGCTGCCGG 60
B     GGCGGCCGCGCGGCACAAACTCAAGATTATGAAACTGCGTGGAGTCAGCCTGGCTGCCGG 60
C     GGCGGCCGCGCGGCACAAACTCAAGATTATGAAACTGCGTGGAGTCAGCCTGGCTGCCGG 60
D     -------------CACAAACTCAAGATTATGAAACTGCGTGGAGTCAGCCTGGCTGCCGG 47
                   ***********************************************

COL   CTGGTTCTTACTGGCCCTGAGTCTTTGGGGGCAGCCCGCAGAGGCTGCGGCTTGCTATGG 107
A     CTGGTTCTTACTGGCCCTGAGTCTTTGGGGGCAGCCCGCAGAGGCTGCGGCTTGCTATGG 120
B     CTGGTTCTTACTGGCCCTGAGTCTTTGGGGGCAGCCCGCAGAGGCTGCGGCTTGCTATGG 120
C     CTGGTTCTTACTGGCCCTGAGTCTTTGGGGGCAGCCCGCAGAGGCTGCGGCTTGCTATGG 120
D     CTGGTTCTTACTGGCCCTGAGTCTTTGGGGGCAGCCCGCAGAGGCTGCGGCTTGCTATGG 107
      ************************************************************

COL   GTGTTCTCCAGGATCAAAATGTGACTGTAGTGGTGTAAAAGGAGAAAAGGGGGAGAGAGG 167
A     GTGTTCTCCAGGATCAAAATGTGACTGTAGTGGTGTAAAAGGAGAAAAGGGGGAGAGAGG 180
B     GTGTTCTCCAGGATCAAAATGTGACTGTAGTGGTGTAAAAGGAGAAAAGGGGGAGAGAGG 180
C     GTGTTCTCCAGGATCAAAATGTGACTGTAGTGGTGTAAAAGGAGAAAAGGGGGAGAGAGG 180
D     GTGTTCTCCAGGATCAAAATGTGACTGTAGTGGTGTAAAAGGAGAAAAGGGGGAGAGAGG 167
      ************************************************************

COL   ATTTCCGGGTTTGGAAGGCCATCCAGGTTTGCCTGGATTTCCAGGTCCAGAAGGGCCTCC 227
A     ATTTCCGGGTTTGGAAGGCCATCCAGGTTTGCCTGGATTTCCAGGTCCAGAAGGGCCTCC 240
B     ATTTCCGGGTTTGGAAGGCCATCCAGGTTTGCCTGGATTTCCAGGTCCAGAAGGGCCTCC 240
C     ATTTCCGGGTTTGGAAGGCCATCCAGGTTTGCCTGGATTTCCAGGTCCAGAAGGGCCTCC 240
D     ATTTCCGGGTTTGGAAGGCCATCCAGGTTTGCCTGGATTTCCAGGTCCAGAAGGGCCTCC 227
      ************************************************************
```

Fig. 4.3. Continued

```
COL   AGGGCCTCGGGGACAAAAGGGTGATGATGGAATTCGAGGGCCACCAGGACCAAAAGGGAT 287
A     AGGGCCTCGGGGACAAAAGGGTGATGATGGAATTCGAGGGCCACCAGGACCAAAAGGGAT 300
B     AGGGCCTCGGGGACAAAAGGGTGATGATGGAATTCGAGGGCCACCAGGACCAAAAGGGAT 300
C     AGGGCCTCGGGGACAAAAGGGTGATGATGGAATTCGAGGGCCACCAGGACCAAAAGGGAT 300
D     AGGGCCTCGGGGACAAAAGGGTGATGATGGAATTCGAGGGCCACCAGGACCAAAAGGGAT 287
      ************************************************************

COL   CAGAGGTCCTCCTGGACTTCCTGGATTTCCAGGGACACCGGGTCTTCCTGGAATGCCAGG 347
A     CAGAGGTCCTCCTGGACTTCCTGGATTTCCAGGGACACCGGGTCTTCCTGGAATGCCAGG 360
B     CAGAGGTCCTCCTGGACTTCCTGGATTTCCAGGGACACCGGGTCTTCCTGGAATGCCAGG 360
C     CAGAGGTCCTCCTGGACTTCCTGGATTTCCAGGGACACCGGGTCTTCCTGGAATGCCAGG 360
D     CAGAGGTCCTCCTGGACTTCCTGGATTTCCAGGGACACCGGGTCTTCCTGGAATGCCAGA 347
      ***********************************************************

COL   CCATGATGGGGCCCCAGGACCTCAAGGTATCCCTGGATGCAATGGAACCAAGGGAGAACG 407
A     CCATGATGGGGCCCCAGGACCTCAAGGTATCCCTGGATGCAATGGAACCAAGGGAGAACG 420
B     CCATGATGGGGCCCCAGGACCTCAAGGTATCCCTGGATGCAATGGAACCAAGGGAGAACG 420
C     CCATGATGGGGCCCCAGGACCTCAAGGTATCCCTGGATGCAATGGAACCAAGGGAGAACG 420
D     CCATGATGGGGCCCCAGGACCTCAAGGTATCCCTGGATGCAATGGAACCAAGGGAGAACG 407
      ************************************************************

COL   TGGATTTCCAGGCAGTCCTGGTTTTCCTGGTCTAGAGGGTCCTCCGGGACCTCCTGGGAT 467
A     TGGATTTCCAGGCAGTCCTGGTTTTCCTGGTCTAGAGGGTCCTCCGGGACCTCCTGGGAT 480
B     TGGATTTCCAGGCAGTCCTGGTTTTCCTGGTCTAGAGGGTCCTCCGGGACCTCCTGGGAT 480
C     TGGATTTCCAGGCAGTCCTGGTTTTCCTGGTCTAGAGGGTCCTCCGGGACCTCCTGGGAT 480
D     TGGATTTCCAGGCAGTCCTGGTTTTCCTGGTCTAGAGGGTCCTCCGGGACCTCCTGGGAT 467
      ************************************************************

COL   CCCAGGTATGAAGGGAGAACCAGGTAGTATAATTATGTCATCACTGCCAGGACCAAAGGG 527
A     CCCAGGTATGAAGGGAGAACCAGGTAGTATAATTATGTCATCACTGCCAGGACCAAAGGG 540
B     CCCAGGTATGAAGGGAGAACCAGGTAGTATAATTATGTCATCACTGCCAGGACCAAAGGG 538
C     CCCAGGTATGAAGGGAGAACCAGGTAGTATAATTATGTCATCACTGCCAGGACCAAAGGG 540
D     CCCAGGTATGAAGGGAGAACCAGGTAGTATAATTATGTCATCACTGCCAGGACCAAAGGG 527
      ************************************************************

COL   TAATCCAGGATATCCAGGTCCTCCTGGAATACAAGGCCCAGCTGGTCCCACTGGTTTACC 587
A     TAATCCAGGATATCCAGGTCCTCCTGGAATACAAGGCCCAGCTGGTCCCACTGGTTTACC 600
B     TAATCCAGGATATCCAGGTCCTCCTGGAATACAAGGCCCAGCTGGTCCCACTGGTTTACC 596
C     TAATCCAGGATATCCAGGTCCTCCTGGAATACAAGGCCCAGCTGGTCCCACTGGTTTACC 600
D     TAATCCAGGATATCCAGGTCCTCCTGGAATACAAGGCCCAGCTGGTCCCACTGGTTTACC 587
      ************************************************************

COL   AGGGCCAATTGGTCCCCCAGGACCACCTGGTTTGATGGGCCCTCCTGGTCCACCAGGACT 647
A     AGGGCCAATTGGTCCCCCAGGACCACCTGGTTTGATGGGCCCTCCTGGTCCACCAGGACT 660
B     AGGGCCAATTGGTCCCCCAGGACCACCTGGTTTGATGGGCCCTCCTGGTCCACCAGGACT 656
C     AGGGCCAATTGGTCCCCCAGGACCACCTGGTTTGATGGGCCCTCCTGGTCCACCAGGACT 660
D     AGGGCCAATTGGTCCCCCAGGACCACCTGGTTTGATGGGCCCTCCTGGTCCACCAGGACT 647
      ************************************************************
```

Fig. 4.3. Continued

```
COL   TCCAGGACCAAAGGGGAATATGGCTTAAATTTCCAGGGACCCAAAGGTGAAAAAGGTGA 707
A     TCCAGGACCAAAGGGGAATATGGCTTTAATTTCCAGGGACCCAAAGGTGAAAAAGGTGA 720
B     TCCAGGACCAAAGGGGAATATGGCTTAAATTTCCAGGGACCCAAAGGTGAAAAAGGTGA 716
C     TCCAGGACCAAAGGGGAATATGGCTTAAATTTCCAGGGACCCAAAGGTGAAAAAGGTGA 720
D     TCCAGGACCAAAGGGGAATATGGCTTAAATTTCCAGGGACCCAAAGGTGAAAAAGGTGA 707
      ************************** ********************************

COL   GCAAGGTCTTCAGGGTCCCCCTGGTCCTCCTGGGCAGATCAGTGAACAGAAAAGACCAAT 767
A     GCAAGGTCTTCAGGGTCCCCCTGGTCCTCCTGGGCAGATCAGTGAACAGAAAAGACCAAT 780
B     GCAAGGTCTTCAGGGTCCCCCTGGTCCTCCTGGGCAGATCAGTGAACAGAAAAGACCAAT 776
C     GCAAGGTCTTCAGGGTCCCCCTGGTCCTCCTGGGCAGATCAGTGAACAGAAAAGACCAAT 780
D     GCAAGGTCTTCAGGGTCCCCCTGGTCCTCCTGGGCAGATCAGTGAACAGAAAAGACCAAT 767
      ************************************************************

COL   TGATGTAGAGTTTCAGAAAGGAGATCAGGGACTTCCTGGTGACCGAGGGCCTCCTGGACC 827
A     TGATGTAGAGTTTCAGAAAGGAGATCAGGGACTTCCTGGTGACCGAGGGCCTCCTGGACC 840
B     TGATGTAGAGTTTCAGAAAGGAGATCAGGGACTTCCTGGTGACCGAGGGCCTCCTGGACC 836
C     TGATGTAGAGTTTCAGAAAGGAGATCAGGGACTTCCTGGTGACCGAGGGCCTCCTGGACC 840
D     TGATGTAGAGTTTCAGAAAGGAGATCAGGGACTTCCTGGTGACCGAGGGCCTCCTGGACC 827
      ************************************************************

COL   TCCAGGGATACGTGGTCCTCCAGGTCCTCCAGGTGGTATGAAAGGTGAGAAGGGTGAACA 887
A     TCCAGGGATACGTGGTCCTCCAGGTCCTCCAGGTGGTATGAAAGGTGAGAAGGGTGAACA 900
B     TCCAGGGATACGTGGTCCTCCAGGTCCTCCAGGTGGTATGAAAGGTGAGAAGGGTGAACA 896
C     TCCAGGGATACGTGGTCCTCCAGGTCCTCCAGGTGGTATGAAAGGTGAGAAGGGTGAACA 900
D     TCCAGGGATACGTGGTCCTCCAGGTCCTCCAGGTGGTATGAAAGGTGAGAAGGGTGAACA 887
      ************************************************************

COL   AGGAGAGCCAGGCAAAAGAGGTAAACCGGGCAAAGATGGAGAGAATGGCCAACCAGGAAT 947
A     AGGAGAGCCAGGCAAAAGAGGTAAACCGGGCAAAGATGGAGAGAATGGCCAACCAGGAAT 960
B     AGGAGAGCCAGGCAAAAGAGGTAAACCGGGCAAAGATGGAGAGAATGGCCAACCAGGAAT 956
C     AGGAGAGCCAGGCAAAAGAGGTAAACCGGGCAAAGATGGAGAGAATGGCCAACCAGGAAT 960
D     AGGAGAGCCAGGCAAAAGAGGTAAACCGGGCAAAGATGGAGAGAATGGCCAACCAGGAAT 947
      ************************************************************

COL   TCCAGGTTTACCTGGTGATCCTGGTTACCCTGGTGAACCAGGAAGGGATGGAGAAAAGGG 1007
A     TCCAGGTTTACCTGGTGATCCTGGTTACCCTGGTGAACCAGGAAGGGATGGAGAAAAGGG 1020
B     TCCAGGTTTACCTGGTGATCCTGGTTACCCTGGTGAACCAGGAAGGGATGGAGAAAAGGG 1016
C     TCCAGGTTTACCTGGTGATCCTGGTTACCCTGGTGAACCAGGAAGGGATGGAGAAAAGGG 1020
D     TCCAGGTTTACCTGGTGATCCTGGTTACCCTGGTGAACCAGGAAGGGATGGAGAAAAGGG 1007
      ************************************************************

COL   CCAAAAAGGTGACATTGGCTCAACTGGGCCTCCTGGACTTGTAATTCCTAGGGACTACAA 1067
A     CCAAAAAGGTGACATTGGCTCAACTGGGCCTCCTGGACTTGTAATTCCTAGGGACTACAA 1080
B     CCAAAAAGGTGACATTGGCTCAACTGGGCCTCCTGGACTTGTAATTCCTAGGGACTACAA 1076
C     CCAAAAAGGTGACATTGGCTCAACTGGGCCTCCTGGACTTGTAATTCCTAGGGACTACAA 1080
D     CCAAAAAGGTGACATTGGCTCAACTGGGCCTCCTGGACTTGTAATTCCTAGGGACTACAA 1067
      ************************************************************
```

Fig. 4.3. Continued

```
COL  GGACGACGACGACAAGGTAACTGTAGGAGAGAAAGGAAACATGGGGTTACCTGGCTTGCC 1127
A    GGACGACGACGACAAGGTAACTGTAGGAGAGAAAGG---AAATATGGGGTTACCTGAGAC 1137
B    GGACGACGACGACAAGGTAACTGTAGGAGAGAAAGG---AAATATGGGGTTACCTGAGAC 1133
C    GGACGACGACGACAAGGTAACTGTAGGAGAGAAAGG---AAATATGGGGTTACCTGAGAC 1137
D    GGACGACGACGAC----TAATCTTGAGTTTGTGCCG---CGGGCGGCCGCTCGTTGAGAC 1120
     *************    ***   *  *   *    *         *           * *
```

Fig. 4.3. Alignment of sequences from Top10 cells to *COL4A5*. Four plasmid sequences (A, B, C and D) have been aligned to *COL4A5* (abbreviated COL). The alignments match perfectly until base 1080 of *COL4A5*. The start codons are highlighted in yellow.

Due to the above problems with TOP10 cells, EC100 cells were chosen for subsequent use. These cells were used by Blue Heron Biotechnologies to propagate the original of pUCminus vector with the *COL4A5* insert, and therefore should work for the transfer into pcDNA3.1/CT-GFP-TOPO. After the full length cDNA has been inserted into this vector, further *in vitro* expression studies will be conducted to ensure that the protein is properly made.

Although this work has been hindered by some difficulties, this is often the case when working with a collagen gene. These genes are large complex, and expression of the protein is often difficult to optimize in *in vitro* experiments. However, because of the precedents set by other groups (Heikkila et al., 2001; Harvey et al., 2003), we are confident that our goal to produce a functional *COL4A5* cDNA that is suitable for *in vivo* gene transfer experiments can be achieved.

CHAPTER V

ANALYSIS OF TYPE IV COLLAGEN GENE EXPRESSION IN AUTOSOMAL RECESSIVE AND X-LINKED FORMS OF HEREDITARY NEPHROPATHY IN THE ENGLISH COCKER SPANIEL AND MIXED BREED DOG

Overview

AS and HN refer to hereditary glomerular nephropathies caused by mutations in type IV collagen genes. These diseases occur in the human (AS) and various breeds of the domestic dog (HN). Among dogs, a mixed breed kindred, termed NAV dogs, has XLHN due to a mutation in *COL4A5*. Additionally, the ECS has ARHN caused by an uncharacterized mutation in either *COL4A3* or *COL4A4*. To assess type IV collagen mRNA levels in kidneys of such dogs, RNA was isolated from renal cortex and examined by real time quantitative RT-PCR (qRT-PCR). This was done for (1) normal NAV male, XLHN-affected NAV male and XLHN-carrier female NAV dogs, (2) ARHN-affected ECS and (3) control dogs, all lacking signs of renal diseases, representing four other breeds. XLHN-affected NAV dogs exhibited a greater than two fold reduction in *COL4A2* and *COL4A5* mRNA levels (P=0.13 and 0.03, respectively). XLHN-carrier female NAV dogs exhibited a greater than two reduction in *COL4A4* and *COL4A5* mRNA levels (P=0.03 and 0.06, respectively). ARHN-affected ECS exhibited a greater than two fold reduction in *COL4A4* and *COL4A6* mRNA levels (P=0.13 and 0.36, respectively). Importantly, for *COL4A5,* the gene causative for XLHN, there was a reduced level of mRNA in XLHN-affected and carrier NAV dogs. This pattern was mimicked to a lesser extent in ARHN-affected ECS *COL4A4* mRNA levels. Given the

similar patterns of *COL4A5* and *COL4A4* mRNA levels in the respective affected dogs, it is likely that a mutation in *COL4A4* is causative for ARHN in the ECS.

Introduction

AS and HN refer to progressive hereditary glomerular nephropathies caused by mutations in three members of the type IV collagen gene family that encode proteins required for normal GBM structure and function. These conditions occur in the human (AS) and various breeds of the domestic dog (HN) and are transmitted in X-linked, autosomal recessive and autosomal dominant fashions.

XLHN has been identified in two families of the dog: 1) a Samoyed kindred and 2) a kindred of a mixed breed dogs, the NAV dogs (Bernards and Valli, 1977; Lees et al., 1999). In the Samoyed model of XLHN, the causative mutation is a single base substitution in exon 35 of *COL4A5*, which results in a stop codon (Zheng et al., 1994). The causative mutation in NAV dogs is a ten base pair deletion in exon nine of *COL4A5* (Cox et al., 2003). ARHN occurs in the English Cocker Spaniel (ECS). The mutation causative for ARHN in the ECS is unknown, but is suspected to be in either *COL4A3* or *COL4A4,* as in human ARAS (Mochizuki et al., 1994; van der Loop et al., 2000; Longo et al., 2002).

Characterization of differences in transcript levels that occur in these two canine models of HN will enhance our understanding of the disease processes. Although mRNA profiles in other animal models of HN have been reported, no previous studies have utilized qRT-PCR. Through the use of qRT-PCR, we identified differences in mRNA

levels of the type IV collagen genes of XLHN-affected, XLHN-carrier and ARHN-affected dogs, compared with dogs having no signs of renal disease. These data suggest that *COL4A4* is the gene causative for ARHN in the ECS.

Materials and methods

Animals

The NAV dogs used in this study were members of a colony maintained at Texas A&M University. Status of normal and XLHN-affected NAV dogs was determined by genotype using primers designed to amplify the region harboring the ten base pair deletion in *COL4A5* (Forward 5′-CGCTTGACTATTTTGTGTGTCATAA-3′, Reverse 5′-AAGGTGATGCTGTGATCTGATTTA-3′). However, the XLHN-carrier female NAV dogs were born prior to implementation of this test (however, one could do the PCR test on these dogs as well), and the status of these dogs was determined by IF staining of their epidermal basement membranes, as previously described (Lees et al., 1999). XLHN-affected dogs were necropsied between eight and ten months of age, when their serum creatinine levels were ≥ 5.0 mg/dL. During necropsy whole kidneys were harvested and renal cortex isolated. Normal NAV dogs were euthanized, whole kidneys harvested and renal cortex isolated, at eight and 15 months of age. XLHN-carrier female NAV dogs were euthanized (for reasons unrelated to HN) and necropsied between 13 months and seven years of age. Whole kidneys were harvested and renal cortex isolated from the XLHN-carrier females in an identical fashion to the normal and XLHN-affected NAVs. ECS used in this study were individual client-owned dogs and were

studied because they developed juvenile-onset renal failure caused by ARHN. Diagnosis of ARHN in affected ECS was confirmed by electron microscopic and IF evaluations of their GBM. Affected ECS were euthanized at ESRD, between seven and nine months of age, and the renal cortex obtained. Because ECS were client-owned animals, kidneys from normal dogs of this breed were not available. Dogs (from several other breeds) not affected with AS, were also used for this study. These dogs were both client-owned and from an unrelated research colony and included two Dalmatians, one German Shepherd Dog, one Great Dane and one Havanese (Table 5.1). These dogs had no signs of renal disease and were euthanized for reasons unrelated to renal function. For all dogs, renal cortex taken at the time of necropsy was stored in RNAlater (Ambion, Austin, TX) until isolation of RNA.

Table 5.1. Number, age and gender of dogs used to evaluate type IV collagen gene expression. [a] Age of the dogs when they were necropsied and renal cortex was obtained for subsequent RNA isolation.

	Number	Age[a] (months)	Gender
ARHN			
Affected English Cocker Spaniels	3	8-12	F(1), M(2)
XLHN			
Affected mixed breed	3	8-10	M
Dogs without HN			
Normal mixed breed	2	9-15	M
Havanese	1	120	M
Dalmatian	2	1-2	F
German Shepherd Dog	1	unknown	unknown
Great Dane	1	18	M

RNA isolation

Total RNA was isolated from renal cortex using RNA STAT-60 (Iso-Tex Diagnostics, Inc., Friendswood, TX) according to the manufacturer's protocol. The resulting RNA was cleaned using the Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA) and the RNase-Free DNase Set (Qiagen Inc.), to avoid DNA contamination. RNA quality was assessed by one step RT-PCR with β-actin primers (Forward 5′-TGCGTGACATTAAGGAGAAG-3′, Reverse 5′-CTGCATCCTGTCGGCAATG-3′), using the Sigma Enhanced Avian HS RT-PCR kit (Sigma Aldrich, St. Louis, MO). RNA was quantified by spectrophotometry and diluted to 1ng/ul.

Real time quantitative RT-PCR

The mRNA levels for the six type IV collagens and *GAPDH* (used for normalization) were assessed using the BioRad MyiQ Single-Color Real-Time PCR Detection System (BioRad Inc., Hercules, CA). Primers and probes (Table 5.2) were based on the published canine sequence and designed using Primer Express 1.0 (Applied Biosystems, Foster City, CA). When possible, probes were designed to span two exons to eliminate amplification of any DNA that might still be present. The 5′ and 3′ ends of the probes were labeled with 6-FAM (6-carboxyfluorescein) and Black Hole Quencher™, respectively (Biosearch Technologies, Novato, CA). Each 25µl reaction contained 0.2µM of each forward and reverse primer and probe, 12.5µl of 2x QuantiTect Probe RT-PCR Master Mix (Qiagen Inc.), 0.25µl of QuantiTect RT Mix (Qiagen Inc.) and 7ng of RNA. The amplification conditions were: 50° for 30 seconds, 95° for 13 minutes and 30 seconds followed by 45 cycles at 95° for ten seconds, 55° for 30 seconds

and 70° for 30 seconds. In order it ensure reproducibility, each sample was amplified at least in duplicate.

Table 5.2. Type IV collagen primer and probe sequences. Probes are labeled with 6-FAM on the 5′ end and BHQ-1 on the 3′ end.

Gene	Size (bp)	Sequence 5′-3′
COL4A1	74	Forward-CCCCAAAGGACAGCAAGGT Reverse-CACCGTCAAAACCAGGAATACC Probe-CGCCACTATCGAGAGAAGCGAG
COL4A2	79	Forward-TTGGCCTGGAAGGTTATCGT Reverse-TTCCCCTCATCTCCTTTGCTT Probe-AAGGGCAAACAGGCTTTCCC
COL4A3	74	Forward-GAGCCTTATATTAGCAGATGCAC Reverse-TCAGTGGTTTGGCTGTGAATG Probe-TGTGAAGGTCCTACGATTGCCATAG
COL4A4	70	Forward-GGCTACAGTCTGTTATACCTGAAGGA Reverse-AAGACCCCTGCCAGACCAA Probe-AGGAGAAGGCCCACAATCAGGA
COL4A5	170	Forward-GAGCATGGAGCCCCTGAA Reverse-TCGTGTGCATCATGAAGGAATAG Probe-CCAGAGCATCCAGCCATTCATTAG
COL4A6	62	Forward-CCAGGACCTGGGTTTTGCT Reverse-AGTAGATGAAGGGCATGGTGCTA Probe-TCCTGCCTGCCCCGCT
GAPDH	67	Forward-CGGATTTGGCCGTATTGG Reverse-GACAATATCCACTTTGCCAGAGTTAA Probe-CGCCTGGTCACCAGGGC

Analysis

In order to obtain fold changes, analysis was carried out as described by Pfaffl assuming a PCR efficiency (*E*) equal to two (Pfaffl, 2001). All affected and carrier dogs were compared with dogs exhibiting no renal disease. Fold changes greater than two were considered to be indicative of changes in mRNA levels. *P*-values were calculated for each group of dogs using the bootstrap with replacement method (Efron, 1979).

Results and discussion

This study utilized qRT-PCR to analyze type IV collagen transcript levels in AS. Previous work by other investigators included the use of IF staining to assess protein expression as well as northern blotting and RNase protection assays to assess mRNA concentrations (Lees et al., 1998; Lees et al., 1999; Rheault et al., 2004; Zheng et al., 2005). To complement these data, qRT-PCR, a more sensitive method for assessment of transcription, was performed. Data generated using this approach provide additional insight regarding changes in mRNA levels in the renal cortex of HN-affected subjects, and offer a useful comparison of these changes between two different genetic forms of canine HN.

XLHN-affected NAV Dogs

To assess changes in mRNA levels in XLHN-affected NAV dogs at ESRD, comparisons were made to dogs, including normal NAV dogs, with no signs of renal disease. As shown in Figure 5.1, there was a greater than two fold reduction in *COL4A5*

mRNA levels (*P*=0.03) in XLHN-affected NAV dogs. No changes in mRNA levels were detected for *COL4A3*, *COL4A4* and *COL4A6* (*P*=0.65, 0.88 and 0.54, respectively).

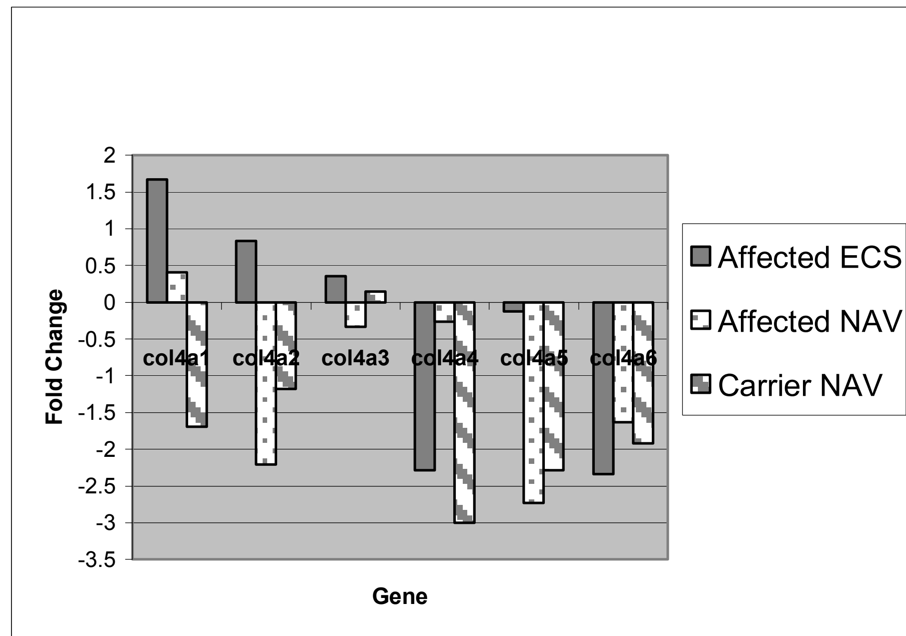

Fig. 5.1. Two fold change in ratio of XLHN-affected and carrier NAV dogs and ARHN-affected ECS compared to normal dogs.

Because the concentrations of full length transcripts of *COL4A5* were reduced in XLHN-affected dogs, and because *COL4A3*, *COL4A4* and *COL4A6* mRNA levels in XLHN-affected dogs were similar to levels in normal dogs, it is tantalizing to hypothesize that the absence of these proteins in renal basement membranes (previously demonstrated by IF (Lees et al., 1999)) is due to a post-translational event. That is, although α3(IV), α4(IV) and α6(IV) are properly translated, the absence of functional α5(IV) proteins prevents the formation of the α3.α4.α5(IV) and α5.α5.α6(IV) trimers and

their subsequent deposition in renal basement membranes. However, further work in necessary to determine the correlations between mRNA and protein levels.

Data for the other type IV collagen genes differed from each other (Fig. 5.1). That is, *COL4A1* exhibited no change in mRNA levels (P=0.69), whereas, *COL4A2*, exhibited a greater than a two fold decrease in mRNA levels (P=0.13). Although the P-value obtained for *COL4A2* is not strongly significant, it does support the trend of a decrease in mRNA levels. This is perplexing because staining for α1(IV) and α2(IV) in the GBM, Bowman's capsule (BC), tubule basement membrane (TBM) and interstitium of XLHN-affected NAV dogs is positive (Lees et al., 1999). However, one could hypothesize that although there is a decrease in mRNA concentration, this is not coupled with an increase in the degradation of the proteins. Because there is an absence of the α3.α4.α5(IV) trimer in all basement membranes, the α1(IV) and α2(IV) proteins are able to be deposited in high enough amounts to be detected by IF staining. Therefore, a plausible explanation for this is that the positive staining results are due to increased retention of these collagens in the basement membranes, as opposed to an increase in their transcription or translation.

Although IF findings for XLHN-affected Samoyeds are similar to those for XLHN-affected NAV dogs (Harvey et al., 1998; Lees et al., 1999), the type IV collagen mRNA profiles for the Samoyed model are noticeably different from those of the NAV dogs. That is, the most recently published data for the Samoyed (Zheng et al., 2005) report an increase in both *COL4A1* and *COL4A2* mRNA levels, a decrease in *COL4A3* and *COL4A4* mRNA levels, no detectable *COL4A5* mRNA and no change in *COL4A6*

mRNA levels. With the exception of *COL4A5* and *COL4A6*, these data differ from the patterns reported here for the NAV dogs. The most striking differences are shown for *COL4A3* and *COL4A4*, for which no changes in mRNA levels were detected in the NAV dogs (Fig. 5.1). However, the findings reported herein do agree with those published for the murine model of XLAS (Rheault et al., 2004).

It is important to note that the mutations leading to XLHN are different for each of these three models. These differences may account for the fact that the XLHN-affected NAV dogs more closely mimic the murine XLAS model than the XLHN-affected Samoyed dogs. Specifically, the XLHN-affected NAV dogs harbor a ten base pair deletion in exon nine of *COL4A5*, where as the XLHN-affected Samoyeds have a nonsense mutation in exon 35 of the same gene (Zheng et al., 1994; Cox et al., 2003). Therefore, the XLHN-affected NAV dogs will have an absence of much of the collagenous domain of the α5(IV) protein, whereas the XLHN-affected Samoyeds will maintain a larger portion of the collagenous domain. The XLAS murine model and the XLHN-affected NAV dogs are similar in both the severity of the mutation and type IV collagen mRNA patterns. This may be due to the fact that the mutation causative for XLAS in the murine model is a nonsense mutation in exon one (Rheault et al., 2004). It has been reported that the more severe the mutation in *COL4A5*, the more severe the disease outcome (Gross et al., 2002), which may explain the differences in transcript levels for these two canine models of XLHN.

XLHN-carrier NAV dogs

Additionally, this work included analysis of type IV collagen transcripts of XLHN-carrier NAV dogs. To date, XLHN-carrier female NAV dogs have been less studied than their affected male counterparts. Those studies involving carriers have remained primarily clinical, although limited IF data are also available. In order to fully understand how the carrier status affects these animals it is important to evaluate their transcript levels. These, along with future analyses, may help to explain the clinical symptoms these carriers exhibit.

Expression of *COL4A5* in XLHN-carrier females is dependent on Lyonization. That is, due to random X-chromosome inactivation, some segments of the GBM will express the allele harboring the mutation, while others will express the wild type allele. Therefore, IF staining for α5(IV), as well as those chains that trimerize with it (*i.e.*, α3(IV), α4(IV) and α6(IV)), is segmental (*i.e.*, mosaic) in all basement membranes in which they normally are found (*i.e.*, the GBM, BC and TBM) in young carrier females .

Like their affected male counterparts, carrier female NAV dogs exhibited a greater than two fold decrease in *COL4A5* mRNA levels (*P*=0.06). *COL4A3* and *COL4A6* levels were also comparable to that of the XLHN-affected dogs, that is, there was no change in mRNA (*P*=0.75 and 0.42, respectively). In direct contrast to affected males, carrier females showed a greater than two fold decrease in *COL4A4* mRNA levels (*P*=0.03) (Fig.5.1). However, the observed relationship between *COL4A4* and the disease may be confounded with animal age. Neither *COL4A1*nor *COL4A2* exhibited a greater than two fold change in mRNA levels (*P*=0.08 and 0.3, respectively) (Fig. 5.1),

but the *P*-value of for *COL4A1* suggests a trend of decreased mRNA levels. Although it is tempting to try to correlate changes in mRNA levels with IF data, this study does not allow for a direct comparison.

Differences in the ages of the dogs and the stages of progression of their renal disease at time of necropsies certainly could contribute to the differences in mRNA levels observed in affected XLHN-male and XLHN-carrier female NAV dogs. The XLHN-carrier females were euthanized for reasons unrelated to ESRD and their ages varied (13 to 84 months), while XLHN-affected males were euthanized because of ESRD at a relatively young age (eight to ten months) (Table 5.1).

ARHN-affected ECS

The ECS samples used for this study were obtained from client owned animals, and this was the reason for the small samples size. Renal tissue from ARHN-affected ECS was difficult to obtain because the dogs must have been diagnosed before they were euthanized, clients had to consent to the use of their dogs for research purposes, and the tissues had to be shipped from various locations. The fact that these dogs are client owned also precluded obtaining normal tissue. This is further complicated by the fact that there is no genetic test to distinguish ARHN-carrier and unaffected ECS. Only parents of affected offspring can be definitively diagnosed as ARHN-carriers.

When compared to dogs without signs of renal disease (including normal NAVs), ARHN-affected ECS at ESRD showed a greater than two fold decrease in *COL4A4* mRNA levels, similar to the decrease in *COL4A5* mRNA seen in the XLHN-affected

NAV dogs (Fig.5.1). The established *P*-value for *COL4A4* of 0.13 is not strongly significant but indicates a trend of decreased mRNA levels. *COL4A6* exhibited a greater than two fold change in its transcript levels (Fig. 5.1), however, a *P*-value of 0.36 was obtained. No provocative changes were observed for *COL4A1*, *COL4A2*, *COL4A3* and *COL4A5* (*P* = 0.23, 0.48, 0.49 and 0.91, respectively) (Fig. 1).

Transcript concentrations differed in the ARHN-affected ECS from that of the XLHN-affected NAV dogs for *COL4A1* and *COL4A2*. That is, the XLHN-affected NAV dogs showed no change in *COL4A1* mRNA levels but did show a greater than two fold increase in *COL4A2* mRNA levels (*P*=0.13). These differences could be due to the fact that these dogs have different genetic backgrounds, and more importantly that they exhibit two forms of HN. This could lead one to hypothesize that different pathways for disease progression could be utilized for the different forms of the disease.

The IF findings for ARHN-affected ECS are similar, in some respects, to those for XLHN-affected NAV dogs. Specifically, there is complete absence of α3(IV) and α4(IV), one of which likely contains the causative mutation, in the GBM and distal tubule basement membrane (dTBM) (Lees et al., 1998) . Of the two genes encoding these proteins, *COL4A4* showed a trend of decreased mRNA levels and *COL4A3* showed no change. Therefore, this suggests that the mutation is present in *COL4A4*. Unlike the NAV dogs, ARHN-affected ECS do have widespread α5(IV) staining in their renal basement membranes (Lees et al., 1998; Lees et al., 1999). This is due to the fact that α5(IV) can trimerize with α6(IV), and in ARHN-affected ECS, these two peptides co-localize in the BC and collecting tubule basement membranes (ctTBM), as well as to

some extent in the GBM. The persistence of α5(IV) in the GBM of ARHN-affected ECS is consistent with the normal levels of *COL4A5* mRNA in these kidneys.

Currently, there exist three murine models of ARHN, two of which are *COL4A3* NC1 domain knockouts and one of which is a *COL4A3/COL4A4* transgenic insertion deleting the 5′ ends of both genes (Cosgrove et al., 1996; Miner and Sanes, 1996; Lu et al., 1999). In those models for which northern blot or IF analysis were performed, the gene that is mutated is absent or mRNA levels are drastically reduced, this is also the case for XLHN-affected NAV dogs (Cosgrove et al., 1996; Miner and Sanes, 1996; Lu et al., 1999). This supports our hypothesis that ARHN in the ECS is due to a mutation in *COL4A4*. However, none of the current murine models knocked out *COL4A4* alone, which together with less quantitative forms of detection, may explain the differences observed in comparison of the ECS with the *COL4A3* knock out murine model described by Miner et al. (Miner and Sanes, 1996). These differences are minor and include the increase in *COL4A1* mRNA levels in the murine model, whereas, there was no significant change in mRNA levels for either *COL4A1* or *COL4A2* in the ARHN-affected ECS (Miner and Sanes, 1996).

This report provides a comprehensive view of type IV collagen transcript concentrations in two canine models of HN. This allows for comparisons between previously published data for other murine and canine models, and also allows a comparison between two forms of HN. While direct comparisons cannot currently be made between IF data and mRNA levels, it is important to understand how these data may relate to each other. Of course, further investigations should be conducted to clarify

this issue. This study also provides a method suitable for investigating other forms of AS which have not yet been characterized, such as ADHN in the Bull Terrier (Hood et al., 1995). Finally, the data provide evidence suggesting that *COL4A4* is the causative gene in the ECS form of ARHN. Further studies are needed to identify the causative mutation.

CHAPTER VI

GENETIC CAUSE OF AUTOSOMAL RECESSIVE HEREDITARY NEPHROPATHY IN THE ENGLISH COCKER SPANIEL*

Overview

Autosomal recessive hereditary nephropathy (ARHN) in the English Cocker Spaniel is caused by a type IV collagen defect, but the underlying mutation is unknown. 134 English Cocker Spaniels (12 with ARHN, eight obligate carriers and 114 others), three mixed breed dogs with X-linked hereditary nephropathy (XLHN) and seven other dogs without hereditary nephropathy were studied to determine the cause of this disease in the English Cocker Spaniels. Diagnosis of ARHN was based on transmission electron microscopy and immunostaining of kidney. Quantitative real time RT-PCR (qRT-PCR) was used to compare *COL4A3*, *COL4A4* and *COL4A5* mRNA levels in renal cortex from ARHN-affected English Cocker Spaniels, XLHN-affected dogs and dogs without HN. The entire coding region of *COL4A4* was sequenced in two ARHN-affected dogs, two obligate carriers, two English Cocker Spaniels of unknown status and two normal mixed breed dogs. The exon containing the mutation was sequenced for all 134 English Cocker Spaniels. Quantitative real time RT-PCR implicated *COL4A4* as the gene harboring the mutation, and sequencing identified a single nucleotide substitution at base 115 as the

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cause of ARHN in English Cocker Spaniels. This mutation, which causes a premature stop codon in exon three of *COL4A4*, segregated with clinical status in all affected dogs and obligate carriers. The mutation was also identified in 39 of 114 other English Cocker Spaniels with previously unknown status. Now that the cause of this disease has been identified, use of a test for the mutation will permit eradication of ARHN in the English Cocker Spaniel.

Introduction

Although terms used to identify the condition have evolved over the years, an hereditary renal disease has been observed in the English Cocker Spaniel since the late 1940s. Krook described a juvenile nephropathy that he termed renal cortical hypoplasia in 40 Cocker Spaniels that were examined at the Royal Veterinary College in Stockholm from 1946 to 1956 (Krook, 1957). He stated that the condition, "as a rule", was characterized by "serious" albuminuria (Krook, 1957). Additional early reports about renal cortical hypoplasia in Cocker Spaniels from Sweden, Switzerland and Australia also described juvenile onset of a fatal proteinuric nephropathy (Persson et al., 1961; Freudiger, 1965; English and Winter, 1979). Subsequent studies demonstrated that the disease was inherited in an autosomal recessive fashion and that the primary renal lesions involved glomeruli (Steward and Macdougall, 1984; Robinson et al., 1985; Macdougall et al., 1987; Koeman et al., 1989). With these developments, familial nephropathy or "FN" became the diagnostic term most widely used for the disease. This term is the name by which most English Cocker Spaniel owners and breeders still know

the disease. However, we believe that hereditary nephropathy is the most appropriate diagnostic term to use for this type of primary glomerular disease in dogs.

Beginning in the 1980s, investigators using transmission electron microscopy noted that the ultrastructural appearance of the glomerular basement membranes (GBM) of affected dogs resembled that of human Alport syndrome (Potter et al., 1985; Macdougall, 1989), but that clue was of little help while the molecular and genetic basis of Alport syndrome remained obscure. Starting in the late 1980s, however, a series of discoveries established that Alport syndrome was caused by type IV collagen defects (Barker et al., 1990; Lemmink et al., 1994; Mochizuki et al., 1994; Hudson et al., 2003). Thus, when distinctive ultrastructural GBM changes were identified in an affected dog examined in 1992 at Texas A&M University, (Lees et al., 1997) we began systematic investigations of the disease based on the hypothesis that it was a type IV collagen disorder. Subsequent studies characterized the clinical, pathologic and ultrastructural features of the disease, including the evolving pattern of abnormalities observed as the disease progressed (Harvey et al., 1998; Lees et al., 1998). Importantly, immunostaining of kidney from affected dogs demonstrated complete absence of the α3(IV) and α4(IV) collagen peptide chains that normally are present in the GBM (Lees et al., 1998). This finding further suggested that the mutation responsible for the disease would affect one of the two genes encoding these proteins.

Each of six type IV collagen genes encodes a distinct α chain (1-6) that can be assembled into three heterotrimers: α1.α1.α2(IV), α3.α4.α5(IV) and α5.α5.α6(IV) (Hudson et al., 2003). In adult kidney, the collagen network that provides the structural

framework for the GBM is composed of α3.α4.α5(IV) heterotrimers. This network is essential for normal GBM structure and function because renal disease, most notably Alport syndrome, ensues when it is absent or abnormal (Harvey et al., 1998; Hudson et al., 2003). Specifically, mutations in any one of the three genes encoding the α3(IV), α4(IV), or α5(IV) proteins can cause Alport syndrome. This occurs because a defect in any of the chains involved in a heterotrimer can prevent its proper assembly. The *COL4A5* gene, which encodes the α5(IV) chain, is located on the X-chromosome in humans and dogs (Hudson et al., 2003; Lowe et al., 2003), and mutations in this gene cause human X-linked Alport syndrome and canine X-linked hereditary nephropathy (XLHN) (Zheng et al., 1994; Knebelmann et al., 1996; Cox et al., 2003; Hudson et al., 2003). The *COL4A3* and *COL4A4* genes, encoding the α3(IV) and α4(IV) chains, respectively, are located on human chromosome two and canine chromosome 25 (Hudson et al., 2003; Lowe et al., 2003). Mutations in either one of these two genes cause autosomal forms of Alport syndrome in humans, the same is suspected to be true of autosomal forms of canine hereditary nephropathy (Lemmink et al., 1994; Mochizuki et al., 1994; Ding et al., 1995; van der Loop et al., 2000; Longo et al., 2002; Vega et al., 2003; Pescucci et al., 2004).

In this report, we describe the use of quantitative real time RT-PCR (qRT-PCR) to identify *COL4A4* as the candidate gene causative for autosomal recessive hereditary nephropathy (ARHN) in English Cocker Spaniels. Sequencing of *COL4A4* identified a single base change in exon three that produces a premature stop codon. This is the first autosomal form of canine hereditary nephropathy in which the underlying genetic cause

has been identified. Appropriate use of a genetic test for this mutation will permit breeders of English Cocker Spaniels to eradicate the disease.

Materials and methods

Dogs and samples

From 1993 through 2005, ARHN was diagnosed at Texas A&M University in 18 English Cocker Spaniels. Each diagnosis was based on finding both the characteristic ultrastructural GBM changes by transmission electron microscopy and the distinctively abnormal pattern of type IV collagen expression in renal basement membranes by immunostaining, as previously described (Lees et al., 1998). Diagnosis of ARHN in individual dogs identified specific families in which the disease trait was inherited, so family histories were collected. Parents of affected dogs were presumed to be obligate heterozygous carriers of the disease trait. Whenever possible, genomic DNA was obtained from affected dogs, each of their parents and as many closely related dogs as were available. Additionally, DNA samples were collected from some English Cocker Spaniels that were not known to be related to any dog in which ARHN had been diagnosed. A commercially available kit, Gentra Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN), was used to isolate DNA from nucleated cells in whole blood or buccal swab specimens, according to the manufacturer's instructions.

Renal specimens suitable for RNA isolation also were obtained at necropsy from 13 dogs (Table 6.1). The three English Cocker Spaniels with ARHN were among the 18 dogs mentioned previously, and the three mixed breed dogs with XLHN were from

kindred maintained in a colony at Texas A&M University. The dogs with XLHN harbor a ten base pair deletion in *COL4A5* (Cox et al., 2003). Status of dogs in the kindred with XLHN was determined by genotype of this mutation with the following primers: Forward 5′-CGCTTGACTATTTTGTGTGTCATAA-3′, Reverse 5′-AAGGTGATGCTGTGATCTGATTTA-3′. The seven dogs without hereditary nephropathy that were studied included two unaffected dogs from the XLHN kindred.

Table 6.1. Number, age and gender of dogs used to evaluate type IV collagen gene expression in samples of renal cortex.
[a] Age of the dogs when they were necropsied and renal cortex was obtained for subsequent RNA isolation.

	Number	Age[a] (months)	Gender
ARHN			
Affected English Cocker Spaniels	3	8-12	F(1), M(2)
XLHN			
Affected mixed breed	3	8-10	M
Dogs without HN			
Normal mixed breed	2	9-15	M
Havanese	1	120	M
Dalmatian	2	1-2	F
German Shepherd Dog	1	unknown	unknown
Great Dane	1	18	M

However, all of the English Cocker Spaniels that we studied were client-owned, and samples of renal cortex from unaffected dogs of this breed were not available. Consequently, we used renal cortical specimens that were available from five dogs of

other breeds. These dogs were both client-owned and from an unrelated research colony and included two Dalmatians, one German Shepherd Dog, one Great Dane and one Havanese. These dogs had no evidence of renal disease and were euthanized for reasons unrelated to renal function. All renal cortex samples obtained at necropsy were stored in RNAlater (Ambion Inc, Austin, TX), an RNA stabilization solution, until isolation of RNA using a RNA STAT-60 following the manufacturers protocol (Iso-Tex Diagnostics Inc, Friendswood, TX).

Additionally, during 2005 and 2006, formalin-fixed specimens of kidney from English Cocker Spaniels with juvenile-onset renal failure were received from Great Britain (one dog) and Sweden (one dog). Before shipment to Texas A&M University, the tissue samples were processed and embedded for routine light microscopic and transmission electron microscopic evaluations. Upon receipt, the embedded tissues were sectioned, stained and examined by standard methods, as previously described (Lees et al., 1998). Family histories were also collected for these dogs, together with DNA specimens from closely related dogs. Again, the Gentra Puregene DNA Isolation Kit (Gentra Systems), a commercially available kit, was used to isolate DNA from nucleated cells in whole blood or buccal swab specimens, according to the manufacturer's instructions.

Quantitative real time RT-PCR

Levels of mRNA transcripts for *COL4A3*, *COL4A4*, *COL4A5* and *GAPDH* (used for normalization) in renal cortex were quantified using the BioRad MyiQ Single-Color

Real-Time PCR Detection System (BioRad Inc, Hercules, CA). Primers and probes (Table 6.2) were designed with Primer Expression 1.0 (Applied Biosystems, Foster City, CA) based on the published canine sequence. The probe designed for each of *COL4A3*, *COL4A4* and *COL4A5* were designed to span two exons to eliminate the detection of any DNA that might still be present. The 5′ and 3′ ends of the probes were labeled with 6-FAM (6-carboxyfluorescein) and Black Hole Quencher™ (Biosearch Technologies, Novato, CA), respectively. Each 25µl reaction contained 0.2µM of each forward and reverse primer and probe, 12.5µl of 2x QuantiTect Probe RT-PCR Master Mix (Qiagen Inc., Valencia, CA), 0.25µl of QuantiTect RT Mix (Qiagen Inc.) and 7ng of RNA. The

Table 6.2. Primer and probe sequences for quantitative real time RT-PCR.

Name	Size (bp)	Sequence 5′-3′
COL4A3	74	Forward-GAGCCTTATATTAGCAGATGCAC Reverse-TCAGTGGTTTGGCTGTGAATG Probe-TGTGAAGGTCCTACGATTGCCATAG
COL4A4	70	Forward-GGCTACAGTCTGTTATACCTGAAGGA Reverse-AAGACCCCTGCCAGACCAA Probe-AGGAGAAGGCCCACAATCAGGA
COL4A5	170	Forward-GAGCATGGAGCCCCTGAA Reverse-TCGTGTGCATCATGAAGGAATAG Probe-CCAGAGCATCCAGCCATTCATTAG

amplification conditions were: 50° for 30 seconds, 95° for 13 minutes and 30 seconds followed by 45 cycles at 95° for 10 seconds, 55° for 30 seconds and 70° for 30 seconds. In order to ensure reproducibility, each dog was amplified in at least duplicate. More

specifically, each dog for *GAPDH* was amplified five times, *COL4A3* and *COL4A5* three times and *COL4A4* five times.

Results were analyzed by comparing ARHN-affected and XLHN-affected dogs with the dogs that did not have hereditary nephropathy. Fold changes were obtained as described by Pfaffl (Pfaffl, 2001), and fold changes greater than two were considered to be indicative of differences in mRNA levels. *P*-values were calculated for each comparison (*i.e.*, ARHN-affected vs. unaffected and XLAS-affected vs. unaffected) using the bootstrap with replacement method (Efron, 1979). This method is performed by resampling with replacement 100 times from the collection of measured values, while honoring the null hypothesis (i.e., that there is no difference between groups' mean responses). *P*-values are then estimated by the fraction of bootstrap values that exceed the actual observed differences, and thus could have been by random chance alone.

Sequencing of COL4A4

The canine *COL4A4* gene contains 47 exons, and the entire coding region was sequenced and analyzed. Most of the coding region (41 exons) was analyzed by amplifying and sequencing individual exons together with their flanking intronic regions. However, three pairs of exons (10 and 11, 13 and 14, and 38 and 39) were sequenced together because of the short length of their intervening introns. Thus, a total of (44) sets of primers were designed to amplify portions of *COL4A4* using the published canine genome sequence (Table 6.3). Intron/exon boundaries were determined by aligning the canine genome sequence in the region on chromosome 25 around

COL4A4 and the previously published mRNA sequence for *COL4A4* (accession No. AY263363).

Amplification by PCR was carried out with each reaction containing 50ng of DNA, 2mM of MgCl, .25mM of each dNTP, 1.0µM of each primer (forward and reverse), 5% DMSO, 0.001mg of Bovine Serum Albumin (Promega, Madison, WI), 0.75 units of *Taq* DNA Polymerase (Fisher Scientific, Pittsburgh, PA), and 1µl of 1X *Taq* DNA Polymerase Buffer B (Fisher Scientific). Amplification cycling conditions were as follows: 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55° - 58°C for 30 seconds and 72°C for 30 seconds, then a single cycle at 72°C for 10 minutes. Products were separated by gel electrophoresis and then visualized using ethidium bromide. When multiple amplification products were present, the desired amplicon was purified using the Qiaex II Gel Extraction Kit (Qiagen Inc.), a commercially available kit. If only the desired amplicon was present, 7µl of the product was purified using ten units of Exonuclease I (Epicentre, Madison, WI) and 1 unit of Shrimp Alkaline Phosphatase (Roche, Indianapolis, IN) and incubated at 37°C for 30 minutes and followed by a 15 minute incubation at 80°C. Purified products were then used for nucleotide sequencing using the Big Dye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems) and resolved on an ABI 3730 Genetic Analyzer (Applied Biosystems).

To identify the mutation, sequence was obtained from two affected dogs, two obligate carriers, two English Cocker Spaniels of unknown genetic status and two normal mixed breed dogs. Sequences were aligned using Clustal W

(http://www.ebi.ac.uk/clustalw/) and nucleotide polymorphisms that segregated with ARHN in English Cocker Spaniels were identified. To verify the mutation, exon three was amplified and sequenced in every affected English Cocker Spaniel and obligate carrier from which an adequate DNA sample was available.

Once the causative mutation was identified, exon three was amplified and sequenced in 114 additional English Cocker Spaniels having unknown genetic status. This was accomplished using the same primers for exon three (Table 6.3) and nucleotide sequencing procedure as has already been described.

Results

Quantitative real time RT-PCR

To assess gene expression changes in XLHN-affected dogs and ARHN-affected English Cocker Spaniels, comparisons were made with dogs having no evidence of renal disease. There was a greater than two-fold reduction in *COL4A5* mRNA (P=0.03) in XLHN-affected dogs (Fig 6.1), but no changes in mRNA levels for *COL4A3* or *COL4A4* (P =0.65 and 0.88, respectively) were detected. English Cocker Spaniels with ARHN had a greater than two-fold reduction in *COL4A4* mRNA levels, similar to that seen for *COL4A5* in XLHN-affected dogs; however, the P-value for this reduction (P=0.13) was not significant. No provocative changes in mRNA levels were observed for *COL4A3* or *COL4A5* (P =0.49 and 0.91, respectively) (Fig 6.1). This pattern of findings for the English Cocker Spaniels with ARHN suggested that underlying mutation was more likely to be in *COL4A4* than in *COL4A3*, so we proceeded with sequencing of that gene.

Table 6.3. Primers and melting temperatures (^{o}C) used for PCR amplification of canine *COL4A4*.

Exon	Tm	Sequence
1	55	F: CAGGGCATAGAACCTCACTTA R: CTGCTGTGCTCTGGACATTAG
2	55	F: TCACTAATGACAGCAGCCTAT R: ACCTGGGTAACTTGGTAAGAA
3	55	F: CCCTCTCACCAAGCCAC R: GTTGCTGACTGTTGTTAGATGTT
4	55	F: GTTTGTGTTAGAAGAGAGCG R:CATAGTAGTGCTGGTGAGTGG
5	55	F: GCTCATTATTTATGTTTTCAAG R: AAGCACAGTAGGGAGAGGG
6	57	F: GAGTCACCAT TGCCATAACG R: CAGCCTCCTCCCACAGTCT
7	55	F: GAAATCTCCACTAGCGAAAC R: GCAAGAACAGTTAGGAGATACT
8	57	F: CCACACAGCCTTCCACAGTT R: ACCCAGGTAATGCCAAATGAT
9	55	F: GATGTTTCTGGGACTGTGAT R: ACTGGTAATGGGAGGTGTA
10 and 11	55	F: GAACCCAGGGCAACC R: TTAACATCTGCTCCTCCAT
12	55	F: GCCACGCAGGATTGTATG R: GCTGAGGTTGCTTTGGG
13 and 14	55	F: GAAGAGATAATGTCTGAAAGATGTA R: CCCAGGTGCCCCAATA
15	55	F: GCCATAAAGCAGTTTCATAAG R: ATCTGTAAAATAAATGTGTCTCC
16	55	F: ATGCGATACTGAGATTTTGC R: GATACGAGGTGATCCCCA

Exon	Tm	Sequence
17	55	F: GTCGGATTCCTTTGTCATTC R: CCACCCAAGTCCCATCTC
18	55	F: CAGTGCTGCTCCAAGTTC R: GGTGAGGGTGAGGCTGTC
19	55	F: CGGTTTCCATTTGTGTGC R: CAGGCTTCATAGAACTGTTTG
20	55	F: CTTAGAGAGAAAGAGTCATAGGAA R: AGGAGTGCTCATAGGCGTA
21	55	F: CCCCCCAACAGACCAT R: CAGCACTGAGAACAGCACC
22	55	F: AGGTCAAGAGCCTCAGTTTTAT R: GAAATGTGAACAGCAAGGAATA
23	55	F: GTCCTGTGTTTCCTCCTACT R: CCAAAGATGGCTCTGATTA
24	55	F: GGTTTGCTATTGAGTAACTGTCTA R: TTATTGAACGGTTCTGCTGTA
25	55	F: AGGCAGTTCAAATCGTCTC R: AACTATTGGTTCATCATCTTAC
26	55	F: AGGCGAGGCAACAGTTACATA R: CCCTGGACCACCTGCTTAC
27	55	F: CAAGGTGGCAAAGCAAC R: GCATTCTACATTTCTAAGGC
28	55	F: CGTCGGTTGCTGGTACT R: GCTACTTGTCATTCTGTGGAG
29	55	F: GATGGATGTTGCTTCGTG R: GGATGGACAGTATCAGGCT

Table 6.3 Continued

Exon	Tm	Sequence
30	55	F: GTCCCACATCAGACTTCCT R: CTAAAGCAGACACCAGCAA
31	55	F: TACTGTGCTGATACTGTGCTG R: GCTGGAACTGGTATTAGATGT
32	55	F: TATGGCTTAGGGCAGGAA R: AAGGGCAATGATGTTTACAGA
33	55	F: CACCTCTAATACTGGAGTTGTA R: ATGCTAAATGTGCGTGCT
34	55	F: TGAAGATAAACTATAAAGACAAAT R: TGGAGCCCAACACAAG
35	55	F: CAAGGGCTGAAGTTGGAGGTT R: GAGGGATGGGTAGGTCTGAGTG
36	55	F: AGGTCTAGGGGATAAAAGTG R: CTGAGTGAGAGAGAAGAGGAA
37	55	F: GAGCGTGTTAAATAATAGCCA R: TCATCTTCAGTCCTAATAATAGTCC
38 and 39	55	F: GCAGCAGGTGGTTGGTCTCAGCA R: CCACCTGCCGCATTGA
40	55	F: CCCTTCATCTCTCGCTTGC R: GAACTTGTGTTTCTTCCCCTTAC
41	55	F: TGGGTTCAGTCCATCAGA R: GAATAGGGTCCTCACATACAG
42	55	F: GGATGGGGACTTAGTTATGTA R: AAGCACTCACGCTCTGG
43	55	F: GGACTGTTGAGCATTCTTTG R: GCTTACACTGCCCCATACT
44	58	F: CTCGGGCTCAGGGTCTAAC R: GGCTGCGGATCAGTGC
45	55	F: CTCCTCCTCTCTGGCTCC R: TAAAATGTTGATGAATCTGTAAAAT
46	56	F: GGAGGCGTGTCTGTGGGT R: CCGTGTCTCAAGAGGCTATGG
47	55	F: GTTGGTTCTTCCCTGGATAAT R: AACTGGAGTCTGAAATGAGCAC

Sequencing of COL4A4

Regions of *COL4A4* were sequenced from genomic DNA obtained from a total of 134 English Cocker Spaniels. These included 12 affected dogs, eight obligate carriers and 89 other dogs from the United States and Canada, as well as 19 dogs from Great Britain and six dogs from Sweden.

To identify the causative mutation, *COL4A4* was sequenced and analyzed for two ARHN-affected dogs and two obligate carriers. The sequence obtained was compared to that obtained from two English Cocker Spaniels of unknown status and two normal

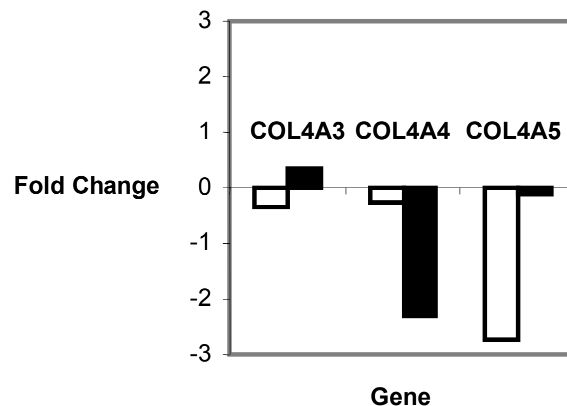

Fig. 6.1. Results of quantitative real time RT-PCR analysis of the expression of selected type IV collagen genes in the renal cortex of XLHN-affected mixed breed dogs and ARHN-affected English Cocker Spaniels. Each bar shows the fold change in the designated group of affected dogs compared with the group of dogs without HN for the indicated gene. White bars represent XLHN-affected mixed breed dogs, black bars represent ARHN-affected English Cocker Spaniels.

mixed breed dogs, as well as to the published canine coding sequence for *COL4A4* (accession AY2633363) and the published 7X NIH genomic reference sequence for the dog. This analysis revealed a single nucleotide substitution (adenine changed to thymine) at base 115, causing a nonsense mutation (lysine changed to a stop codon) in exon three of both affected dogs (Figs 6.2 and 6.3). Additionally, each of the two carriers had both an adenine and a thymine (*i.e.*, an adenine from the normal allele and a thymine from the mutated allele) at base 115 (Fig 6.2 and 6.3).

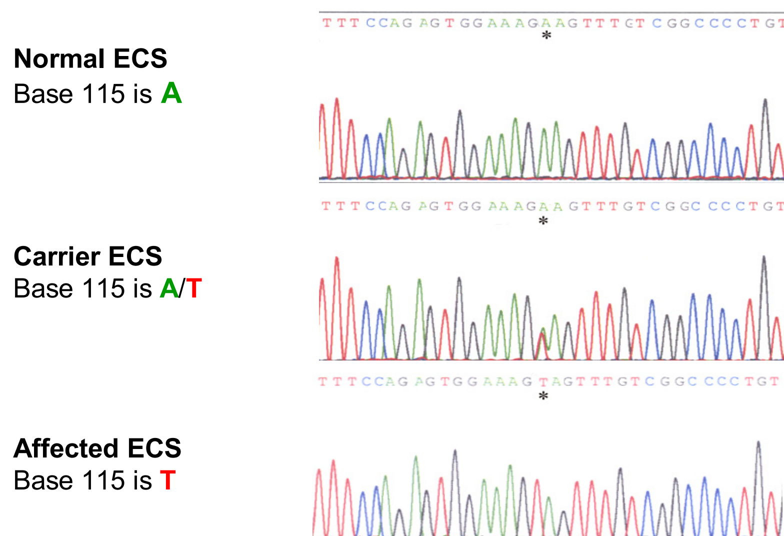

Fig. 6.2. Chromatographs showing DNA sequence results for *COL4A4* in English Cocker Spaniels (ECS). Top panel, a normal dog; middle panel, an obligate carrier of ARHN; and bottom panel, an ARHN-affected dog. The affected dog is homozygous and the carrier is heterozygous for the mutated allele (thymine instead of adenine) in exon three. Base at which mutation occurs is marked by an asterisk in each panel.

To verify the mutation, exon three of *COL4A4* was sequenced using genomic DNA from 12 English Cocker Spaniels with ARHN, as well as from eight obligate carriers. In every instance in which an affected or obligate carrier dog's DNA was sequenced and analyzed, the same mutation was identified (*i.e.*, homozygous in the affected dogs and heterozygous in the carriers).

Sequence analysis of *COL4A4* also revealed two single nucleotide polymorphisms (SNPs) that co-segregated with the disease. Both SNPs are in the intron upstream of exon 42. The first, which is a thymine to adenine substitution, is located 93 bases upstream. The second, which is a thymine to cytosine substitution, is 90 bases upstream. These two nucleotide changes do not alter the coding sequence of the gene, but they are inherited with the disease trait.

Exon three of *COL4A4* was also sequenced using genomic DNA from 114 English Cocker Spaniels having unknown genetic status. These dogs included 65 that were close relatives of obligate carriers (*i.e.*, parents, siblings, or offspring) in which the random odds of finding the dog to be heterozygous for the mutation were one in two (*i.e.*, 50% chance of also being a carrier). Among these 65 dogs, 34 (52.3%) were heterozygous for the mutation and were thus also identified as carriers. Among the remaining 44 dogs, in which the chances of being a carrier were ≤ 25%, only 3 (6.8%) were heterozygous for the mutation. Five dogs (two of which were identified as carriers) could not be placed into the above categories due to the absence of relationship information or a conclusive diagnosis. Therefore, a total of 39 previously unknown carriers were identified.

Light microscopic examination of stained sections of formalin-fixed, paraffin-embedded kidney from the English Cocker Spaniel in Sweden that died of juvenile-onset renal failure was sufficient to exclude ARHN as the cause of that dog's renal disease. The histologic lesions were unmistakably those of renal dysplasia. Samples of DNA were available from the affected dog, both of its parents and three other closely related dogs. Tests for the mutated *COL4A4* allele that causes ARHN were negative in these six dogs.

Results of the pathologic evaluations of kidney from the English Cocker Spaniel from Great Britain that was suspected to have ARHN were inconclusive. The light microscopic and transmission electron microscopic findings were compatible with the disease, but they were not sufficiently distinctive to permit definitive diagnosis. Glomerular ultrastructure was too poorly preserved in the available formalin-fixed tissue for critical evaluation, and the material was not suitable for immunostaining. However, DNA specimens were available from six related dogs, including the dog's sire. Genetic testing demonstrated that the sire was heterozygous for the mutated *COL4A4* allele and that the other five dogs were homozygous for the normal allele.

Overall, the sequence data we obtained for exon three of *COL4A4* in English Cocker Spaniels identified the genetic status of a total of 134 dogs from the United States, Canada, Great Britain and Sweden. With 12 affected dogs and 47 carriers in this sample, the overall frequency of the mutated allele in the English Cocker Spaniels that we studied was 0.2649 (71 of 268). However, this sample included at least four separate kindreds, and in two small kindreds (containing 19 and six dogs) in which an affected

dog had not been diagnosed, the frequencies of the mutated allele were 0.0263 (one of 38) and zero (none of 12), respectively. However, this frequency may not be accurate for the ECS population as a whole because only four kindred were studied.

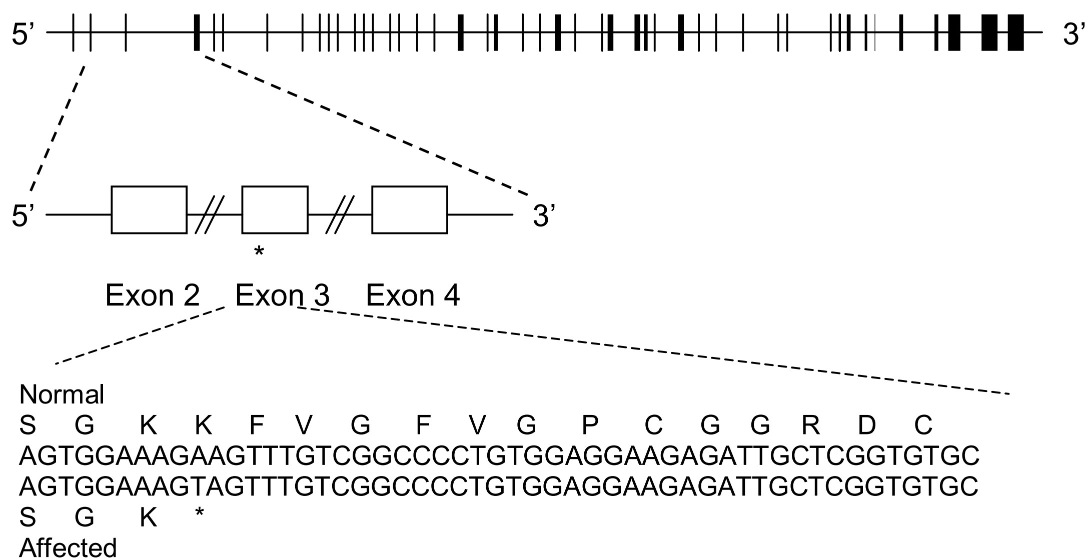

Fig. 6.3. Diagram of the *COL4A4* mutation that causes ARHN in the English Cocker Spaniel. The top panel is a schematic representation of genomic *COL4A4*, indicating all 47 exons and the intervening introns in approximate scale. The middle panel represents an enlargement of the portion of the top panel that includes exons two, three and four. The bottom panel shows a portion of the nucleotide and translated amino acid sequences within exon three (base 106 – 157) for normal dogs and for affected dogs. The A-T transversion at base 115 (marked by the asterisk) creates a premature stop codon in the affected dog sequence.

Discussion

Hereditary nephropathy is caused by mutations in any one of the three genes encoding the type IV collagens found in the GBM. Specifically, XLHN is caused by

mutations in *COL4A5* and mutations in either *COL4A3* or *COL4A4* can be causative for ARHN. Results of our qRT-PCR analysis of mRNA transcript levels for these genes in the renal cortex of affected dogs showed that in the XLHN-affected dogs, *COL4A5*, the gene harboring their causative mutation, had a greater then two-fold reduction. However, *COL4A3* and *COL4A4* showed no change in mRNA levels. In ARHN-affected English Cocker Spaniels, mRNA levels for *COL4A4*, but not for *COL4A3* or *COL4A5*, also were reduced greater than two-fold. Based on the observation that the only reduction in mRNA levels for XLHN-affected dogs was in the gene harboring the mutation, and that ARHN-affected English Cocker Spaniels had a reduction in only one of the three transcripts as well, we hypothesized that the cause of ARHN in English Cocker Spaniels was most likely a *COL4A4* defect. We then succeeded in identifying the causative mutation by sequencing that gene.

Predictable consequences of the missense mutation we identified in exon three of *COL4A4* explain all of the salient features of ARHN in affected English Cocker Spaniels. The mechanism by which mutations causing HN lead to the previously discussed changes in mRNA levels for their respective genes is uncertain, but nonsense-mediated decay is a potential explanation (Baker and Parker, 2004). Another feature of ARHN in affected English Cocker Spaniels is that immunostaining demonstrates total absence of α4(IV) chains and α3(IV) chains in GBM, together with greatly reduced GBM α5(IV) chain expression (Lees et al., 1998). Because the mutation creates a premature stop codon, any protein synthesis directed by the mutated gene would generate a severely truncated peptide that would be unable to combine with its normal

partners (*i.e.*, α3(IV) and α5(IV) chains) to produce stable α3.α4.α5(IV) heterotrimers. Without the ability to form α3.α4.α5(IV) heterotrimers, the only collagen networks that can form in renal basement membranes of English Cocker Spaniels with ARHN are composed of α1.α1.α2(IV) or α5.α5.α6(IV) heterotrimers, or both. This accounts for the observation that all renal basement membranes in English Cocker Spaniels with ARHN that exhibit α5(IV) chain expression by immunostaining also exhibit co-expression of α6(IV) chains, but renal expression of both α3(IV) chains and α4(IV) chains is totally absent even where α5(IV) chains are expressed (Lees et al., 1998). Finally, absence of the α3.α4.α5(IV) network in the GBM of affected dogs accounts for all of the ultrastructural, clinical and pathologic features of the progressive nephropathy that occurs in English Cocker Spaniels with ARHN. The nephropathy that develops in these dogs has essentially the same ultrastructural, clinical and pathologic features as the nephropathy that occurs in dogs with XLHN in which the α3.α4.α5(IV) network is missing from the GBM because of a *COL4A5* mutation (Jansen et al., 1986; Jansen et al., 1987; Thorner et al., 1987; Harvey et al., 1998; Lees et al., 1999).

We expect that the mutation causing ARHN in English Cocker Spaniels will be identical by descent within this breed worldwide. To date, however, the only nations from which we have had opportunities to test dogs that were found to carry the mutated allele are the United States, Canada and Great Britain. Assessment of the distribution and frequency of the mutated allele among English Cocker Spaniels worldwide will require further studies.

The fact that ARHN has persisted in the English Cocker Spaniel breed for more than 50 years is a testament to the difficulties that have previously existed in identifying carriers of the mutated allele. In the past, carriers have been accurately identified only when a mating produced at least one affected dog that was properly diagnosed. Thus, although astute breeders commonly managed to avoid producing affected dogs, the mutated allele readily persisted in the gene pool and unsuspected carriers eventually were mated to one another from time to time. Availability of a test for the mutated *COL4A4* allele should solve these problems.

In English Cocker Spaniels with renal disease, a test for the mutated *COL4A4* allele will be useful only in those dogs with clinical and light microscopic findings that are compatible with a diagnosis of ARHN. Therefore, even in a young English Cocker Spaniel with renal disease, testing for the mutated allele should be done in conjuction with a thorough clinical and pathologic investigation of the dog's illness. First, a genetic diagnosis of ARHN should be assessed for its ability to adequately explain the dog's clinical and pathologic findings. Additionally and even more importantly, absent an appropriate clinical and pathologic investigation, a genetic test that excludes ARHN provides no information about what disease actually caused the nephropathy.

These concepts are illustrated by our evaluations of the specimens in this study from the English Cocker Spaniels in Great Britain and Sweden. The clinical and pathologic features of the dog from Great Britain were consistent with ARHN, but the condition could not be definitively diagnosed using the formalin-fixed tissues that were available for examination. In this setting, results of the genetic test permitted us to

resolve our uncertainty about the correct diagnosis. Conversely, pathologic features of the nephropathy in the dog from Sweden were sufficient to exclude ARHN as its cause and tests for the mutated allele were negative. The final diagnosis, however, was based on clinical and histopathological evaluations.

A test for the mutated *COL4A4* allele may have some utility as an aid in the diagnosis of ARHN in English Cocker Spaniels with renal disease, but it will be of greatest value in guiding future selective breeding of these dogs. Indeed, proper use of a test to detect the mutated allele should permit breeders of English Cocker Spaniels to eradicate ARHN from this breed in as short a time as one generation and without having to abandon any breeding lines that have other desirable traits. This can be accomplished by testing all dogs in the breeding population to ensure that matings of two carriers are avoided so that no more dogs will develop renal failure due to ARHN. Testing of progeny from matings that include one carrier can be used for selection of future breeding stock to completely eliminate the mutate allele from the English Cocker Spaniel gene pool within the foreseeable future.

CHAPTER VII

CONCLUDING REMARKS

The work reported herein describes research conducted using a canine model of human AS. By using the dog as a model, a better understanding of this disease in the human can be obtained. However, this research will also directly benefit the dog. This work is a result of the effort of many participants with the goal of better understanding HN in the dog and AS in the human.

As a continuation of the discovery of the mutation causative for XLHN in the NAV dogs, Chapter II describes the development of a genomic test to accurately genotype these dogs. It was only through the publication of the canine genome that this goal was accomplished. This genomic test allows dogs to be genotyped with speed and accuracy never before available. Early knowledge of the genotype also allows for better planning for future experiments.

The importance of studying XLHN-affected NAV males is clear but it is also, critical to study the female carriers of this disease. The original idea for this research was to use the XLHN-carrier NAV females to study the effect X-chromosome inactivation patterns have on disease progression. However, in the course of our studies, it was determined that most XLHN-carrier females have very similar patterns of unskewed XCI. Although these dogs are not suitable for a long-term study to correlate disease progression with XCI, XCI patterns found in renal tissue do accurately reflect those found in blood. This provides further evidence that invasive biopsies are not necessarily needed to study XCI in this disease, peripheral blood will suffice.

Currently, there are no treatments available to substantially prolong the lives of AS or HN patients without dialysis or transplantation. Therefore, in an effort to help in the development of a gene transfer therapy, a synthetic canine *COL4A5* cDNA was constructed. In the future, this gene will be introduced in to affected kidneys in an attempt to repair the GBM. Proof that this type of therapy is possible would ideally lead to the development of a similar treatment for humans.

Quantitative real time RT-PCR was used to analyze type IV collagen gene expression patterns of XLAS-affected NAV dogs and ARHN-affected ECS. The primary objective of this work was to identify the gene harboring the mutation causative for ARHN in the ECS. By comparing patterns of mRNA expression of these dogs, it was hypothesized that *COL4A4* harbored a mutation that is causative for ARHN in the ECS.

Data from the quantitative real time RT-PCR experiment indicated that *COL4A4* should be sequenced. Sequencing revealed a nonsense mutation in *COL4A4* of exon three in affected dogs. This mutation causes a severely truncated protein. The identification of this mutation has allowed a test to be developed that allows owners of ECS to identify dogs that are carriers. Prior to this test, the only way to positively identify carriers of this disease was by the production of affected offspring. Now, breeders can arrange to mate carriers only with normal dogs and, in this way, keep the desired traits of the breed while avoiding the production of affected pups.

In conclusion, this work provides more insight into HN in the domestic dog while also moving towards the goal of a gene transfer treatment for humans affected by

AS. Identification of the mutation causative for ARHN in the ECS is the finding necessary for elimination this disease from the breed.

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