12-2012

CHARACTERIZATION OF TWO TYPE IV COLLAGENS INVOLVED IN AUTOSOMAL RECESSIVE HEREDITARY NEPHROPATHY

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CHARACTERIZATION OF TWO TYPE IV COLLAGENS INVOLVED IN AUTOSOMAL RECESSIVE HEREDITARY NEPHROPATHY

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Genetics

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by
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December 2012

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ABSTRACT

The glomerular basement membrane (GBM) of the kidney is highly specialized and required for filtration of waste products from the bloodstream. One of the major structural and functional units of the GBM is a network composed of type IV collagen heterotrimers, or protomers. Early in development of the human and dog, an isoform switch occurs, and the α1α1α2 (IV) network found in the nascent GBM is replaced with α3α4α5 (IV) protomers. Mutations in any of the genes encoding proteins found in the mature GBM network may prevent protomer formation and cause a progressive disorder leading to end stage renal failure (ESRD). In the human, the disease is known as Alport syndrome (AS) and is characterized by proteinuria, hematuria, and in half of affected patients, deficits in hearing. The GBM undergoes characteristic ultrastructural changes, and immunostaining of renal samples reveals a loss of α3 (IV) and α5 (IV) proteins with a corresponding increase in α1 (IV) chains. Hereditary nephropathy (HN) is the canine counterpart of AS and has been reported in different breeds. Affected dogs display clinicopathological signs identical to those described in AS patients, with the exception of hearing loss. There were two major objectives for this project. The first objective was to amplify and sequence a region previously lacking coverage on CFA25 between the genes encoding the α3 (IV) and α4 (IV) chains. This was necessary in order to span gap regions in the canine reference genome. The second objective was to identify the mutation causative for HN in a breed not previously reported to present with the disease.

DEDICATION

For Harley, Mom, and Dad

ACKNOWLEDGMENTS

I would first like to thank my advisor Dr. Keith Murphy for giving me the opportunity to work in his lab as a doctoral student and providing me with the means to carry out the research described in this dissertation. I am especially grateful to Dr. Leigh Anne Clark for taking over as chair of my committee and allowing me to join her lab for my last semester. I wish to express sincere thanks to Dr. Alison Starr-Moss for being a wonderful mentor for the past four years. I would also like to thank my advisory committee, Dr. Meredith Morris and Dr. Jim Morris, for their support.

I am grateful to the other members of the Canine Genetics Laboratory at Clemson University for their friendship and help, especially Dr. Kate Tsai, Rooksana Noorai, and Caitlin Rinz. Additionally, I would like to acknowledge Dr. Christopher Saski and Jeanice Troutman of the Clemson University Genomics Institute and Dr. George Lees of Texas A&M University for their guidance and assistance along the way.

My greatest thanks go to my husband and my family. Harley, your unconditional love and support have carried me through the past two years, and for you I am truly grateful. Mom, Dad and Matt, you've always been my biggest cheerleaders, providing encouragement every step of the way. None of this would have been possible without you.

TABLE OF CONTENTS

Page

Table of Contents (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

The Dog as a Model

The domestic dog, *Canis lupus familiaris*, is a remarkable model for genetic studies. More than 400 breeds of dog exist, carefully developed and selected for specific morphological and behavioral characteristics that define individual breeds. Depending on the governing organization, the number of recognized breeds varies from as few as 175 (American Kennel Club; http://www.akc.org/breeds/index.cfm) to 343 (Fédération Cynologique Internationale; http://www.fci.be/presentation.aspx). Most breeds have a relatively recent origin, having existed for only the past 250 years (Ostrander *et al*. 2008; Parker *et al*. 2010). For a dog to be purebred, both of a dog's parents must be registered members of the same breed. Each breed therefore represents a closed population with gene flow limited by the so-called "pedigree barrier" (Mosher *et al*. 2009; Patterson. 2000).

Within a breed, genetic diversity can be restricted by factors such as population bottlenecks due to catastrophic events or breed popularity, the small number of founders used to create a breed, and the popular sire effect (Ostrander and Kruglyak. 2000). A consequence of these factors is an increased presence of recessive alleles within a population. Carriers of such alleles are asymptomatic, and disease alleles can therefore be transmitted through many generations before detection. Inbreeding to maintain

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This dissertation follows the style of *Genetics*

desired traits thus results in a high frequency of autosomal recessive diseases (Ostrander. 2012; Patterson. 2000).

Although limited genetic diversity within a breed can be unfortunate for canine health, it is this very fact that makes the dog so useful as a model for study of human hereditary diseases and other traits that are governed genetically. Within human populations, mutations in a large number of loci may be responsible for a single disease. However, because of the high degree of intrabreed genetic similarity, often the analogous disease in the dog is caused by a mutation at a single locus (Mosher *et al*. 2009). In addition, the extensive linkage disequilibrium found within a breed allows for the mapping of a disease locus using less than 10% of the markers required to map the same locus in human populations (Lindblad-Toh *et al*. 2005; Sutter and Ostrander. 2004).

Dogs present with more hereditary diseases than any other non-human animal, and nearly half of these affect only one or a few breeds (Patterson. 2000). More than 360 hereditary diseases are documented in the dog, and of these, nearly 300 are considered models of human diseases (Mosher *et al*. 2009; http://omia.angis.org.au). Often, the diseases are caused by defects in the same human and canine genes; thus, identification of the causative mutations is equally important to veterinary medicine and human medicine (Parker and Ostrander. 2005).

The dog is an excellent model organism because large numbers of offspring can be produced in a relatively short amount of time, and extensive pedigree records are maintained by breeders and owners. Humans share their homes, activities and sometimes diet with their dogs; dogs and humans are therefore exposed to many of the same

environmental factors (*e.g.*, pollution, chemicals) that can affect onset of diseases, clinical presentations and the rates of progression (Shearin and Ostrander. 2010). Medical surveillance including preventive care and management of illnesses, and compilation of detailed health and treatment records for dogs, is second only to that provided to humans (Ostrander *et al*. 2008). Another advantage of the dog is comparable organ sizes to those of the human. From a genetic perspective, a critical advantage of the dog as compared to smaller model organisms (*e.g.*, the mouse) is that diseases are naturally occurring and do not have to be induced though knock in and knock out methodologies. Induced disease models do not allow for the study of disease progression and may not accurately portray the symptoms of the modeled diseases. Finally, with the exception of non-human primates, the canine genome displays a greater degree of sequence similarity with the human genome than other model organisms (Shearin and Ostrander. 2010).

The Canine Genome

Several notable advances in canine genomics over the past 15 years have accelerated research for the benefit of the dog and as a model. Specifically, in 2003, a 1.5x coverage sequence of the canine genome was generated by Celera Genomics. Canine orthologs of approximately 75% of identified human genes were represented by reads of at least 100 continuous bases from one or more exons (Kirkness *et al*. 2003). A dense radiation-hybrid gene map was generated based on the survey sequence in order to better resolve the order of identified genes and markers (Hitte *et al*. 2005). Finally, a National Institutes of Health-funded project resulted in a 7.5x sequence derived from a purebred Boxer (Lindblad-Toh *et al*. 2005). The assembled genome is 2.4 giga base pairs,

contains over 19,100 genes, and is estimated to account for 99% of the euchromatic genome (Mosher *et al*. 2009). Following publication of the first build of the canine genome assembly, a White Paper submitted to the National Human Genome Research Institute proposed improvement of the reference genome through shotgun sequencing of larger gaps and primer walking to fill in small- and medium-sized gaps. Approximately 1% of the genome falls within gaps in coverage. On average, gap regions are estimated to contain 57% GC content, likely due to the presence of CpG islands and promoter regions (Blakesley *et al*. 2010).

One such gap is located on CFA25, between two genes encoding members of the type IV collagen family, *COL4A3* and *COL4A4*. The six type IV collagen genes are situated in unique head-to-head arrangements on three chromosomes. Each pair of genes shares a bifunctional promoter (see below for a detailed discussion of type IV collagen). Sequencing of complementary DNA (cDNA) from renal tissue of a Dalmatian determined the nucleotide sequence of the coding region of *COL4A4* and a partial transcript of *COL4A3* (Wiersma *et al*. 2005a). Identification of exon/intron boundaries and comparison with the human orthologs revealed that portions of the first exon of each gene as well as the promoter region likely fall within a coverage gap.

Type IV Collagens

Six genetically distinct α chains, designated α1 (IV) through α6 (IV), make up the collagen (IV) family and are encoded by *COL4A1-COL4A6* respectively. As mentioned previously, these genes are situated in paired head-to-head arrangements on opposite strands of DNA: *COL4A1* and *COL4A2* are on CFA22 (HSA13); *COL4A3* and

COL4A4 are on CFA25 (HSA2); and *COL4A5* and *COL4A6* are located on the X chromosome in both dog and human. A common promoter located between the 5′ regions of each gene pair controls expression, although the mechanisms of expression and regulation are not well-understood. *COL4A2* is theorized to have evolved from a duplication and inversion event of *COL4A1*; subsequent duplications of both genes and their bidirectional promoters gave rise to *COL4A3*/*COL4A4* and *COL4A5*/*COL4A6* (Hudson *et al*. 1993).

Type IV collagens are integral structural components of mammalian basement membranes found in nearly all organs. Two distinct types of α chains exist: α1-like chains (α1, α3 and α5) and α2-like chains (α2, α4, α6). Individual α chains self-assemble into heterotrimers, or protomers, of two α1-like chains and a single α2-like chain. Assembly of α chains occurs in only three possible combinations: α1α1α2 (IV), α3α4α5 (IV) and α5α5α6 (IV). All three chains must be normal for formation of the heterotrimer to occur (Boutaud *et al*. 2000). The α1α1α2 (IV) network is a ubiquitous component of basement membranes throughout the body; however, distribution is tissue-specific for α3α4α5 (IV) (retina, lung, inner ear, testes and GBM of mature kidney) and α5α5α6 (IV) (skin, testes, esophagus, smooth muscle and Bowman's capsule of the glomerulus) (Alexakis *et al*. 2006).

Each α chain has three distinct domains: a short, cysteine-rich 7S region at the amino terminus, a central, helical, collagenous region, and a non-collagenous region (NC1) at the carboxy terminus (Hudson *et al*. 2003; Ortega and Werb. 2002). Of the subregions, the middle collagenous domain is the longest and is composed of interrupted

repeats of a Gly-X-Y motif. X and Y residues are usually proline and hydroxyproline; the cyclic structure of these amino acids causes a slight bend in the α chain, giving collagens their characteristic helical form (Butkowski *et al*. 1987; Fagg *et al*. 1990; Hudson *et al*. 2003; Prockop and Kivirikko. 1995). Glycine is the only amino acid with a side chain (hydrogen) appropriately sized to fit at the center of the assembled protomer (Miner. 2011b). The NC1 domain contains a hypervariable region necessary for correct assembly of α chains into heterotrimers (Sundaramoorthy *et al*. 2002).

In the glomerulus, α chain synthesis is followed by intracellular self-assembly of protomers. Fully-formed heterotrimers are secreted into the extracellular space where they dimerize at their NC1 domains (Abrahamson *et al*. 2009; Sundaramoorthy *et al*. 2002). Each newly-formed dimer then links to three other dimers through their 7S domains to form a tetramer. In this manner, the collagen (IV) network is created. During glomerulogenesis, the collagen (IV) network in the GBM is composed of α1α1α2 (IV) protomers. Shortly after birth, the GBM undergoes a developmental switch in which α3α4α5 (IV) heterotrimers replace the nascent collagen network (Kalluri *et al*. 1997; Miner and Sanes. 1994). Although the reason for the switch is not well-understood, it is hypothesized that α3α4α5 (IV) protomers are more resistant to proteolytic degradation than are α1α1α2 (IV) protomers (Abrahamson *et al*. 2009). Differences in membrane strength between the α1α1α2 (IV) network and the α3α4α5 (IV) network may in part explain the developmental isoform switch and why the nascent collagen network cannot adequately compensate for loss of the mature α3α4α5 (IV) network. Recently, Wyss and colleagues (2011) measured the biomechanical strength of the GBM from normal mice

and from mouse models of early stages of AS, an inherited disease of the type IV collagens (Wyss *et al*. 2011). In the AS mouse model, the GBM retains the α1α1α2 (IV) collagen network in the absence of the α3α4α5 (IV) network (Kang *et al*. 2006). Prior to the occurrence of disease-related fibrogenesis, GBM from AS mice were found to be weaker than membranes of wild-type mice under simulated glomerular pressure conditions (Wyss *et al*. 2011).

Alport Syndrome

AS is a human hereditary disorder affecting basement membranes. The primary symptom is progressive nephropathy, with patients exhibiting increasing hematuria and proteinuria. As glomerular filtration rate declines, the kidneys can no longer effectively remove nitrogenous waste compounds from the blood, thus azotemia is also common (Lemmink *et al*. 1997). Approximately 50% of affected human patients suffer hearing loss and, in rare cases, ocular defects. Depending on the severity of the mutation, affected persons usually reach ESRD by the end of their third decade and require chronic dialysis and/or renal transplant (Atkin *et al*. 1988; Bekheirnia *et al*. 2010). The majority (~80%) of Alport patients have the X-linked form of the disorder (XLAS), caused by mutations in the *COL4A5* gene (Barker *et al*. 1990; Martin *et al*. 1998). Mutations in *COL4A3* and *COL4A4* account for the remainder of AS cases which are inherited in autosomal recessive fashion (ARAS) or, much less frequently, in autosomal dominant manner (ADAS) (Heidet *et al*. 2001; Longo *et al*. 2002; Longo *et al*. 2006; Pescucci *et al*. 2004; van der Loop *et al*. 2000).

The GBM is a unique, double-layered extracellular matrix and is formed from the junction of the endothelial lining of the capillary and the podocyte cell foot processes surrounding the glomeruli. It is composed of three uniform but distinct layers, the thickest being the *lamina densa* in the middle. Under normal circumstances, in conjunction with fenestrated endothelial cell layers, the GBM prevents proteins and blood cells from crossing the capillary wall into the urinary space (Miner. 2012).

In AS patients, the GBM undergoes drastic ultrastructural changes observable by transmission electron microscopy (TEM). The *lamina densa* loses its uniform consistency and causes the GBM to have a "basket weave" appearance (Barsotti *et al*. 2001; Miner. 2011a). Some sections of the GBM become abnormally thick or thin, and podocyte effacement creates gaps on the extracellular capillary surface, which allow albumin to pass through the GBM into the urinary space (Abrahamson *et al*. 2007). Immunofluorescent staining of glomeruli from AS patients reveals an increase in α1 (IV) chains. A lack of, or segmental, staining for α3 (IV), α4 (IV) and α5 (IV) chains is evident in most XLAS-affected males. Renal biopsies from male and female patients with ARAS or ADAS show α5 (IV) staining of the lining of Bowman's capsule only and a complete lack of α3 staining (Barsotti *et al*. 2001; Kashtan and Michael. 1996; Kashtan. 1998).

As mentioned previously, the only treatments for AS are dialysis and renal transplantation. Renal transplants not only carry the risk of graft-versus-host disease, but patients also face the possibility of anti-GBM nephritis (Mojahedi *et al*. 2007). Full length proteins are not produced from genes harboring mutations, and therefore the immune system does not recognize these products as self. The same antigenic regions in the NC1

domains that enable immunostaining also serve as immunogenic targets (Kashtan and Michael. 1996; Mojahedi *et al*. 2007).

Canine Models of AS

Hereditary nephropathy (HN) is a spontaneously-occurring canine analog of AS. Dogs affected by HN suffer from proteinuria and hematuria between four and six months of age. Azotemia is also present and becomes more severe with progression of the disease. Invariably, HN progresses to ESRD between the ages of 12 to 18 months (Jansen *et al*. 1984; Lees *et al*. 1998a; Lees *et al*. 1998b; Lees *et al*. 1999). Clinical signs include anorexia, polydipsia, fatigue and vomiting but often do not present until affected dogs reach ESRD. Deafness, which is characteristic of AS, does not occur in HN-affected dogs (Jansen *et al*. 1986; Lees *et al*. 1998b; Lees *et al*. 1999). The definitive ultrastructural GBM abnormalities observed in renal sections from AS patients are also found in samples from HN-affected dogs. Podocyte effacement is also observed (Hood *et al*. 2002; Lees *et al*. 1998b; Lees *et al*. 1999; Thorner *et al*. 1996).

Similar to AS, the mode of inheritance of HN in the dog can be X-linked (XLHN), autosomal recessive (ARHN) or autosomal dominant (ADHN). XLHN has been described in both a Samoyed family (Jansen *et al*. 1984; Jansen *et al*. 1986) and a mongrel kindred termed Navasota (NAV) dogs (Lees *et al*. 1999). In the Samoyed, a G to T substitution in exon 35 of *COL4A5* was found to cause a premature stop codon (Zheng *et al*. 1994); affected NAV dogs harbor a 10-base pair deletion in exon 9 of *COL4A5* believed to cause a truncated protein from a frameshift mutation in exon 10 (Cox *et al*.

2003). Immunostaining indicated an absence of α5 (IV) expression in both families as seen in patients with XLAS (Kashtan and Michael. 1996; Lees *et al*. 1999; Zheng *et al*. 1994).

ARHN had a devastating effect on the English cocker spaniel (ECS) breed for over 50 years (Krook. 1957; Lees *et al*. 1998a; Lees *et al*. 1998b; Robinson *et al*. 1985; Steward, AP and MacDougall, DF. 1984). In 2007, the causative mutation was identified as a nonsense mutation in exon 3 of *COL4A4* (Davidson *et al*. 2007). A genetic screening test for the identified mutation was developed to aid breeders in identifying carriers. Use of the test in conjunction with vigilant breeding practices will allow for complete removal of the deleterious allele from the breed (Davidson *et al*. 2007). ARHN has also been recently identified in the English springer spaniel (ESS), a breed closely related to the ECS (Nowend *et al*. 2012). Immunostaining from affected ECS and ESS reveals a complete lack of α3 (IV) chains while α5 (IV) chains are detected only in the lining of Bowman's capsule (Davidson *et al*. 2007; Nowend *et al*. 2012).

Cases of ADHN have been described in the miniature bull terrier (MBT) and Dalmatian (Hood *et al*. 1995; Hood *et al*. 2002). Proteinuria and ocular defects were present in affected MBT; however, hematuria was not observed in all affected dogs. In the GBM, multilamellation and thickening were observed, consistent with other canine models of AS. Onset of ESRD in MBT is more widely variable than in other forms of HN and ranges from several months to ten years (Hood *et al*. 1995). Although the genetic cause of ADHN in MBT has not been identified, *COL4A3*, *COL4A4* and *MYH9* have been eliminated as candidate genes (O'Leary *et al*. 2009). Clinical signs of ADHN in the Dalmatian are similar to those observed in MBT, although glomerular abnormalities are

not as severe and ocular defects were not present. The causative mutations for these breeds are unknown, and immunostaining of GBM did not reveal any difference in any type IV collagen α chains between normal and affected dogs of either breed (Hood *et al*. 2000; Hood *et al*. 2002). The genetic causes of ADHN are expected to be different in the MBT and Dalmatian but all affected animals within a breed are expected to share the same mutation (Hood *et al*. 1995; Hood *et al*. 2002).

The Dog as a Model for Gene Therapy

Although gene therapy is not currently an option for human AS patients, progress has been made toward developing treatment options (Gross *et al*. 2009). The process for effective glomerular vector delivery (Heikkila *et al*. 1996) is quite complicated. Therefore recombinant α5 (IV) chain expression was performed in the bladder of XLHN-affected dogs (Harvey *et al*. 2003). Despite normal bladder function in XLAS- and XLHN-affected individuals, this organ normally expresses α5α5α6 (IV) chains and is easily accessible for adenoviral injection and thus was chosen for gene therapy experiments (Harvey *et al*. 2003). More recently, AS therapies involving stem cell transfer are being developed in ARAS mouse models with promising results (Katayama *et al*. 2008; LeBleu *et al*. 2009). Although mouse models of human disease are not necessarily ideal, novel therapies developed in such models followed by preclinical validation in a dog model will be of great benefit if we are to progress to human trials.

Successful gene therapy in the dog has led to human clinical trials for several hereditary diseases: leukocyte adhesion deficiency-1 (LAD-1) (Bauer and Hickstein. 2000), Lebers congenital amaurosis (Acland *et al*. 2001; Acland *et al*. 2005; Amado *et al*. 2010;

Maguire *et al*. 2008), Gaucher's disease (Novelli and Barranger. 2001), Duchenne muscular dystrophy (Aartsma-Rus *et al*. 2004; Kinali *et al*. 2009; Shimatsu *et al*. 2003), hemophilia A (Powell *et al*. 2003) and hemophilia B (Manno *et al*. 2006; Xu *et al*. 2003). Although human gene therapy trials existed for LAD-1 prior to the initiation of canine research, a lentiviral vector construct conferring less genotoxicity than previously used viral vectors was developed using in a canine model of LAD-1 (Bauer *et al*. 2011; Nelson *et al*. 2010). In addition, the treatment of Leber's congenital amaurosis using gene therapy led to the initiation of a human clinical trial (Maguire *et al*. 2008), and results of therapy developed for achromatopsia appear promising for future human trials (Komaromy *et al*. 2010).

This project had two major objectives. The first objective was to sequence the first exons and shared promoter region of *COL4A3* and *COL4A4* to fill in the gap in the reference sequence of CFA25. The second objective was to identify and characterize the mutation causative for ARHN in the ESS.

CHAPTER II

SEQUENCING OF THE CFA25 GAP AND 5′ REGION OF CANINE *COL4A3*

Overview

The canine genome reference sequence is an invaluable resource for genetic studies. While the assembly covers 99% of the genome, there are gaps that leave the remaining 1% without coverage. The region between *COL4A3* and *COL4A4* has poor coverage, with gaps that include the first exons of each gene and a shared bifunctional promoter. These genes encode structural basement membrane proteins critical for glomerular filtration and are sites of mutations implicated in hereditary renal disease. To better understand the role of the *COL4A3* and *COL4A4* gene products in kidney disease, it is important to characterize each gene as well as their common promoter. Herein, the identification of the first two exons of *COL4A3* is reported.

Introduction

The canine genome sequence was assembled from whole genome shotgun reads with an average of 7.5x coverage for the majority of the genome. This assembly covers approximately 99% of the euchromatic regions of the genome (Lindblad-Toh *et al*. 2005). It is estimated that just less than 1% of the genome still remains within gaps between assembled contigs. These regions are characterized by higher than average GC content and repetitive sequences (Blakesley *et al*. 2010). Next-generation sequencing technologies have allowed for greater sequence coverage through gap regions; however, in a recent

study, nearly 60% of newly-acquired contigs were not able to be mapped to the reference genome (Kim *et al*. 2012).

A gap in coverage estimated to span approximately 500-base pairs is located on CFA25, between *COL4A3* and *COL4A4*. As with the other type IV collagen pairs, *COL4A1/COL4A2* and *COL4A5/COL4A6*, *COL4A3* and *COL4A4* are situated in a head-to-head arrangement on opposite strands of DNA and share a common bifunctional promoter (Momota *et al*. 1998; Wiersma *et al*. 2005a). Specific protomers composed of three type IV collagen α chains are crucial structural and functional components of the GBM in both the fetal (α1α1α2) and mature (α3α4α5) kidney (Kalluri *et al*. 1997). A defect in any individual gene encoding an α chain prevents formation of the heterotrimer, resulting in degeneration of GBM function and ultimately renal failure (Boutaud *et al*. 2000). To effectively understand the full involvement of the type IV collagens in hereditary renal disease(s), it is necessary to have the full sequences available of each gene pair, and also the associated promoter regions.

In an effort to identify the genetic variation responsible for an inherited nephropathy in the Norwegian Elkhound, Wiersma and colleagues (2005) sought to characterize the mRNA sequences for *COL4A3* and *COL4A4* (Wiersma *et al*. 2005a; Wiersma *et al*. 2005b). Using 5′ rapid amplification of cDNA ends (RACE), the sequence of the coding and 3′ untranslated regions of *COL4A4* were identified (GenBank accession number AY263363.1). A partial mRNA sequence of *COL4A3* was determined through primer-walking techniques (GenBank accession number AY263362.1); however, the identified coding region lacked the 5′ untranslated region and a transcription start site

(Wiersma *et al*. 2005a). Comparison with human *COL4A3* intron/exon boundaries using GENATLAS (http://genatlas.medecine.univ-paris5.fr/) indicated the missing regions corresponded to exon 1 and partial exon 2.

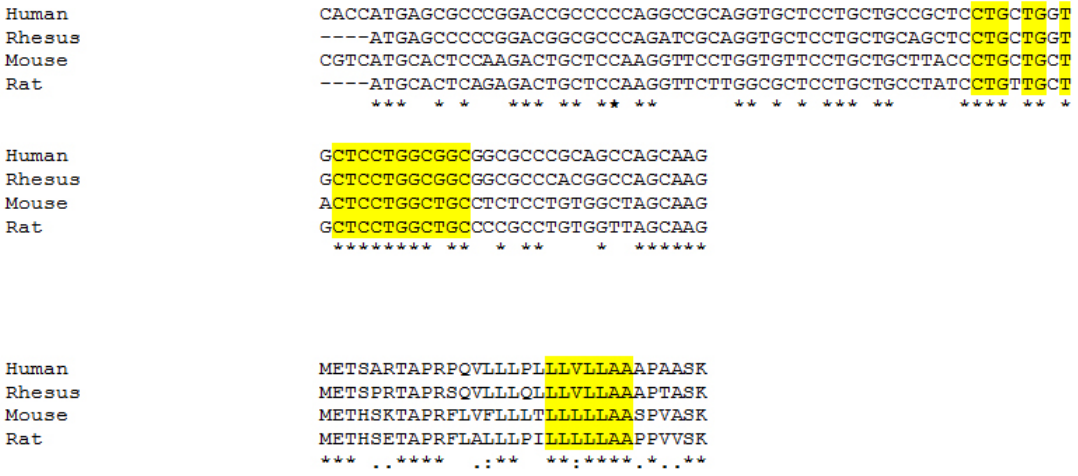

Figure 2.1 Multispecies alignment of *COL4A3* exon 1. The nucleotide alignment is in the upper region. "*" indicates identical bases in all four species. The protein alignment is in the lower region. "*" indicates exact matches, ":" indicates conservative substitutions, and "." indicates semi-conservative substitutions. A highly conserved region used to design a degenerate primer (4A3-DF2) is highlighted in yellow.

The objective of this study was to amplify and sequence the promoter and the 5′ end of *COL4A3*. The human coding region of *COL4A3* shares 86% identity at the nucleotide level and 83% identity at the amino acid level with the canine ortholog and thus is a useful reference for primer design and sequence verification (Wiersma *et al*. 2005a). Alignment of published sequences of *COL4A3* exon 1 from human and three additional mammals by ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) indicated regions of high conservation at both the nucleotide and protein levels (Figure 2.1).

Degenerate primers were designed based on regions of exon 1 showing the highest conservation between all four organisms. Through amplification and direct sequencing optimized for GC-rich regions, partial coding sequences of exons 1 and 2 of canine *COL4A3* were identified and validated by comparison to the published human *COL4A3* sequence.

Materials and Methods

Amplification and Sequencing

To amplify the first exon of *COL4A3*, degenerate forward primers were designed based on the consensus sequence from a multispecies alignment of exon 1 and paired with reverse primers annealing to a region of the coding sequence corresponding to exons 6 and 7 (GenBank accession number AY263362.1). All primer sequences are listed in Table 2.1. Each 25 μl reaction contained 2.5 μl 10X *Taq* DNA polymerase buffer B (Fisher Scientific, Pittsburgh, PA), 50 pmol of each primer, 0.2 mM of each dinucleotide triphosphate, 1M betaine (Sigma-Aldrich; St. Louis, MO), 5% dimethylsulfoxide, 1.5 mM magnesium chloride and 2.0 units of *Taq* DNA polymerase (Fisher Scientific; Pittsburgh, PA). Complementary DNA reverse transcribed from clinically normal renal RNA was used as template for all reactions. Amplification was performed under the following conditions: 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, and a single cycle of 72°C for 10 minutes.

PCR products were assessed by agarose gel electrophoresis. Products of correct sizes were excised from the agarose and purified using a commercially available kit (5 PRIME; Gaithersburg, MD). Purified amplicons were sequenced using Big Dye

Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems; Foster City, CA) and resolved on an ABI 3730 Genetic Analyzer (Applied Biosystems; Foster City, CA).

Table 2.1 Primers and melting temperatures (°C) used for polymerase chain reaction

	Primer Name	Sequence	Tm
COL4A3	4A3-DF2*	CTGYTGSTRCTCCTGGCK	58.1
	4A3-R14	CCTGTGGTCCAGGCAAGC	59.2
	4A3-R15	CAGGAGAACCAGCAGGTCCA	59.1
Gap Region	COLP-F	GCACCAACAAAAAATCTGGGCAC	65
	4A3-R16	CGCCAGGAGCACCAACAG	64.7
	4A3-DF3*	ATGAGCSCCCGGACSGCS	71.5
	COLP-R	CAGGAACACGAACAGCAGCATC	65

*Primer contains degenerate bases. Standard International Union of Biochemistry (IUB) nomenclature rules were used in designating degenerate bases: Y (T, C); S (G,C); R (A,G); K (G,T).

Subsequent reactions were based on methods described by Musso and colleagues (2006) to amplify GC-rich templates using PCR additives (Musso *et al*. 2006). Amplification was performed using primers based on experimental sequence obtained above paired with primers flanking the gap region (Table 2.1). Each reaction contained 100 ng of canine genomic DNA, 2.5 μl 10X *Taq* DNA polymerase buffer B (Fisher Scientific, Pittsburgh, PA), 10-50 pmol of each primer, 0.2 mM of each dinucleotide triphosphate, 1.3 M betaine (Sigma-Aldrich; St. Louis, MO), 5% dimethylsulfoxide, 50 μM 7-deaza-dGTP, 2.5 mM magnesium chloride and 1.25 units of *Taq* DNA polymerase (Fisher Scientific; Pittsburgh, PA) in a total volume of 25 μl. Cycling conditions were 94°C for 5 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds; followed by a single cycle of 72°C for 5 minutes.

Reaction products were isolated and purified as described above. Direct sequencing reactions using a 3:1 mix of BigDye Terminator v3.1 Cycle Sequencing and dGTP BigDye Terminator v3.0 Reaction Ready Cycle Sequencing kits (Applied Biosystems; Foster City, CA) were carried out by the Clemson University Genomics Institute. Sequencing results were resolved on an ABI 3730 Genetic Analyzer (Applied Biosystems; Foster City, CA).

Sequence Analysis

Sequencing data were analyzed using Ridom TraceEdit sequencing software (Ridom, Inc., Würzburg, Germany). Canine sequences obtained from amplification using degenerate PCR were aligned with the 5′ coding regions of the human *COL4A3* mRNA sequence (GenBank accession number NM_000091.4) and the previously mentioned partial canine *COL4A3* mRNA to verify identity.

Results and Discussion

In general, promoter regions are GC-rich due to the presence of CpG islands. Because of the proline content in the Gly-X-Y motif found in the collagenous domain, type IV collagens also have a high GC content (Prockop and Kivirikko. 1995). A number of different methods using PCR additives and GC-optimized cycling were attempted to amplify and sequence the 5′ untranslated and coding regions of both *COL4A3* and *COL4A4* as well as the shared promoter region. The use of PCR enhancers including betaine and DMSO in combination with degenerate primers and increased Mg^{2+} concentration allowed for effective amplification of a short region of *COL4A3* from a GC-rich template. Alignment with the published human *COL4A3* mRNA sequence

showed that the 298-base pair degenerate PCR amplicon contained the last 39 base pairs of *COL4A3* exon 1 and the first 32 base pairs of exon 2 (Figure 2.2). The remaining 227 base pairs of amplified sequence aligned both with *COL4A3* sequencing results obtained previously (Nowend *et al*. 2012) and the published canine reference sequence (data not shown).

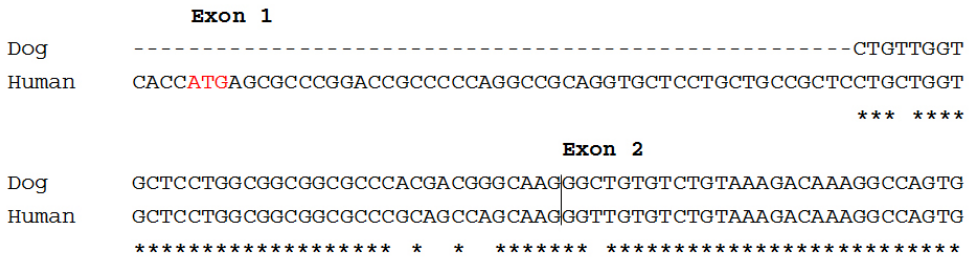

Figure 2.2 Alignment of canine *COL4A3* sequencing results with the published human *COL4A3* 5′ mRNA sequence. Nucleotides corresponding to the start codon in the human sequence are indicated in red. Exons 1 & 2 are labeled and separated by a vertical black line. "*" indicates identical nucleotides in both sequences.

Using the methods described above, it was not possible to determine sequences of the promoter, the remaining sequence of *COL4A3*, or the 5′ untranslated region of *COL4A4*. While amplification using primers flanking the gap region produced amplicons, the resulting products proved intractable to direct sequencing, likely due to the high GC content of the region. Amplification using *COL4A3*-specific primers in combination with the gap-flanking primers produced products; however, none of these aligned with published sequences of *COL4A3* exon 1 from human, rhesus, mouse, or rat (data not shown).

Efforts to amplify and sequence across the gap in sequence of CFA25 are ongoing. Determining the full sequences of *COL4A3* and *COL4A4* as well as the shared promoter would contribute to the characterization of hereditary renal diseases in different breeds of dog. In addition, successful sequencing through the undetermined region of CFA25 would assist in efforts to read through the gaps in the canine genome and improve this genomic tool.

CHAPTER III

CHARACTERIZATION OF THE GENETIC BASIS FOR AUTOSOMAL RECESSIVE HEREDITARY NEPHROPATHY IN THE ENGLISH SPRINGER SPANIEL*

Overview

Autosomal recessive hereditary nephropathy (ARHN), a fatal juvenile-onset renal disease, was diagnosed in two English Springer Spaniels (ESS) based on transmission electron microscopy and immunostaining of kidney. A closely related breed, the English Cocker Spaniel (ECS), has been affected by ARHN for more than 50 years. Recently, the causative mutation of ARHN in the ECS was identified. The ESS does not have the ECS mutation, and so additional genetic analyses were performed. Expression levels of *COL4A3, COL4A4* and *COL4A5* in the renal cortex were determined using quantitative reverse transcription polymerase chain reaction (qRT-PCR) for ARHN-affected dogs and 7 other dogs for comparison. qRT-PCR indicated reduced mRNA transcript expression of both *COL4A3* and *COL4A4* in the kidney of ARHN-affected ESS when compared to those of unaffected dogs. The coding regions of *COL4A3* and *COL4A4* were sequenced for both ARHN-affected dogs and an unaffected dog. Sequencing revealed a single nucleotide substitution in *COL4A4* at base 2806. This results in a premature stop codon in

*Portions of this chapter are reprinted with permission from "Characterization of the Genetic Basis for Autosomal Recessive Hereditary Nephropathy in the English Springer Spaniel" by K. Nowend, A. Starr-Moss, G. Lees, B. Berridge, F. Clubb, C. Kashtan, M. Nabity and K. Murphy, 2012. *Journal of Veterinary Internal Medicine,* 26, 294-301, Copyright 2012 by John Wiley & Sons, Inc.

exon 30. Direct sequencing of exon 30 was done for 63 ESS. Fourteen related dogs were identified as carriers of the mutation, including both parents of the affected dogs. C2806T is a novel mutation causing ARHN in the ESS, a breed not previously reported to be affected by ARHN.

Background

Hereditary nephropathy (HN) is a progressive fatal renal disease that has been identified in several domestic dog breeds or kindreds. The disease was first described as renal cortical hypoplasia (Krook. 1957), and also has been called familial nephropathy (Lees *et al*. 1998a; Lees *et al*. 1998b; Robinson *et al*. 1985; Steward, AP and MacDougall, DF. 1984) and hereditary nephritis (Hood *et al*. 1995; Hood *et al*. 2002; Lees *et al*. 1998b) in various published accounts. HN is the result of glomerular basement membrane (GBM) defects that alter GBM structure and function in the mature kidney. The GBM is a sheet-like layer of extracellular matrix that lies between and supports the endothelial and visceral epithelial cell components of the glomerular capillary walls. This structure is primarily composed of heterotrimers of laminins and type IV collagens (α1-α5 chains). During glomerular development, the GBM is initially composed of α1α1α2(IV) heterotrimers, but as the glomeruli mature, α3α4α5(IV) heterotrimers gradually replace the α1α1α2(IV) network. Moreover, normal synthesis and assembly of α3α4α5(IV) heterotrimers is crucial for maintaining the structure and function of the GBM in adult kidney. Defects in any of the genes encoding α3(IV), α4(IV), or α5(IV) collagen chains can cause the GBM to develop distinctive abnormalities during adolescence that initiate a progressive renal disease culminating in juvenile-onset chronic renal failure (Khoshnoodi

et al. 2008). In humans, the nephropathy caused by type IV collagen defects is usually called Alport syndrome and is associated with certain hearing and ocular abnormalities in many affected individuals. In both humans and dogs, type IV collagen-related renal disorders have both X-linked and autosomal patterns of inheritance. This is because the *COL4A5* gene is on the X-chromosome and the *COL4A3* and *COL4A4* genes are on an autosome (chromosome 2 in humans and chromosome 25 in dogs) in both species. For these reasons, *COL4A3, COL4A4* and *COL4A5* are candidate genes for identifying the causative mutations in subjects with HN.

An autosomal recessive form of HN (ARHN) has occurred in the English Cocker Spaniel (ECS) for more than 50 years (Krook. 1957; Lees *et al*. 1998a; Lees *et al*. 1998b; Robinson *et al*. 1985; Steward, AP and MacDougall, DF. 1984). Affected dogs typically develop severe (end-stage) renal disease by the time they are 6 to 24 months of age. In dogs with ARHN, clinical signs often are not observed until late in the course of disease (Lees *et al*. 1998a; Lees *et al*. 1998b), although proteinuria invariably develops a few months before other clinicopathologic changes can be detected. There is no cure for HN; all currently available treatments are symptomatic or supportive in nature. Previous work identified the mutation in *COL4A4* that causes ARHN in ECS (Davidson *et al*. 2007). Efforts to eliminate the disease from the ECS breed using genetic testing coupled with careful breeding practices (i.e. preventing the breeding of 2 carriers) are ongoing and have been highly successful.

The English Springer Spaniel (ESS) is closely related to the ECS and, according to the American Kennel Club, originally came from the same litters (http://www.akc.org).

Despite common ancestry, ARHN has not been described previously in the ESS breed. This report describes the discovery of ARHN in a family of ESS and subsequent identification of a novel *COL4A4* mutation in this kindred.

Materials and Methods

Clinical and Pathologic Evaluations

Two female ESS littermates were evaluated at the Texas A&M University College of Veterinary Medicine and Biomedical Sciences (TAMU-CVM) for renal disease that was first detected when the dogs were 7 months old. Each dog's renal disease was observed to progress to a near-terminal stage over the next 2-3 months, whereupon each dog was euthanized.

Clinical examinations, laboratory testing, and diagnostic imaging of the 2 dogs were performed by conventional methods. Pathologic evaluations of kidney obtained immediately after euthanasia included histologic, ultrastructural, and immunostaining examinations utilizing methods similar to those previously described (Lees *et al*. 1998b). Formalin-fixed tissue was routinely embedded in paraffin and sections cut 3 microns thick were prepared with H&E, PAS, Jones' methenamine silver, and Masson's trichrome stains for light microscopic examination. Cortical tissue for transmission electron microscopic examination was fixed in chilled 3% glutaraldehyde, further processed, sectioned and stained by conventional methods (Lees *et al*. 1998b), and examined in a JEOL TEM-1230 transmission electron microscope. Immunolabeling of renal basement membranes for their content of certain collagen IV proteins was performed with indirect immunofluorescence staining methods using monoclonal antibodies specific for α1/2

chains (MAB M3F7), α3 chains (MAB A2), or α5 chains (MAB A7), as previously described (Lees *et al*. 1998b).

Samples for Molecular Genetic Evaluation

Whole blood and kidney tissue were collected from both affected ESS. DNA was extracted from whole blood using a commercially available kit (Gentra Puregene Blood Kit; Qiagen Inc., Valencia, CA), and renal tissue was stored in an RNA stabilization solution (RNA*later*; Applied Biosystems/Ambion, Austin, TX). Additional blood samples were collected from 25 related ESS (including both parents) and 1 unrelated ESS owned by the breeder. Subsequently, DNA was isolated from buccal cell samples from 35 ESS, unrelated to the kindred, obtained from owners.

RNA was extracted from the kidney tissues of the 2 affected ESS, as well as from archived frozen kidney tissue from 2 ARHN-affected ECS, 2 XLHN-affected mixed-breed (NAV) dogs (Cox *et al*. 2003) and 3 mixed-breed dogs unaffected by any form of HN. Reverse transcription was performed to synthesize complementary DNA (cDNA) from the RNA. The ESS were client-owned; breed-matched tissue samples from unaffected dogs were not available. DNA and RNA quantification was determined using a spectrophotometer (NanoDrop; Fisher Scientific, Pittsburgh, PA).

Genomic Screening of* COL4A4 *Exon 3

Exon 3 of *COL4A4* was amplified by polymerase chain reaction (PCR) to screen for the previously identified ARHN mutation (Davidson *et al*. 2007) using genomic DNA from the 2 affected ESS and 3 ECS with known clinical status: 1 ARHN-affected dog, 1 ARHN-carrier and 1 unaffected dog. Each reaction contained 12.5 µl 2x all-inclusive

PCR premix (ReddyMix Master Mix; AbGene, Rochester, NY), 0.25 μM of each primer and 50 ng of DNA, brought to a final volume of 25 μl with sterilized water. Primer sequences and cycling conditions have been published previously (Davidson *et al*. 2007). Product sizes were verified using agarose gel electrophoresis. The PCR amplicons were treated with 0.5 units of Exonuclease I (New England Biolabs, Ipswich, MA) and 0.25 units of shrimp alkaline phosphatase (Promega, Madison, WI). Purified amplicons then were sequenced (Big Dye Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems, Foster City, CA) and resolved (ABI 3730 Genetic Analyzer; Applied Biosystems, Foster City, CA).

Quantitative Reverse Transcription-PCR (qRT-PCR)

mRNA transcript numbers of *COL4A3*, *COL4A4*, and *COL4A5* were quantified for 9 dogs: 2 ESS and2 ECS with ARHN, 2 NAV dogs with XLHN and 3 dogs unaffected by any form of HN. Primer/probe assays (TaqMan Gene Expression Arrays; Applied Biosystems, Foster City, CA) specific to *C. familiaris* and spanning 2 exons were selected for qRT-PCR. Probes were labeled at the 5′ end with 6-carboxyfluorescein (6-FAM) and at the 3′ end with a nonfluorescent quencher. Each 25 μl reaction contained 12.5 μl 2X QuantiTect Probe RT-PCR Master Mix (Qiagen Inc., Valencia, CA), 0.25 μl QuantiTect RT Mix (Qiagen Inc., Valencia, CA), 0.72 nM of each primer, 0.2 nM of probe and 7 ng of RNA. Reverse transcription, amplification and detection were performed (BioRad iQ5 Real-Time PCR Detection system; BioRad Inc., Heracles, CA) under the following cycling conditions: reverse transcription at 50°C for 30 minutes and inactivation at 95°C for 13 minutes 30 seconds, followed by 45 cycles of 94°C for 15

seconds, 60°C for 60 seconds and 70°C for 30 seconds. All reactions were performed in triplicate. For each sample, quantification of collagen mRNA transcripts was determined by normalization against *β-actin* mRNA transcript numbers, and the magnitude of change between groups was analyzed using the Pfaffl method (Pfaffl. 2001). Any changes in transcript numbers greater than 2-fold were considered significant.

COL4A3 *and* COL4A4 *cDNA Sequencing*

Amplification of the coding regions of *COL4A3* and *COL4A4* was performed for both affected ESS and a normal control dog. RNA was reverse transcribed (High Capacity Reverse Transcription kit; Applied Biosystems, Foster City, CA), and the resulting cDNA was used as a template for amplification. Amplicons were 500-base pairs (bp) long and designed to overlap by approximately 75 bp at each end, spanning the coding regions for both genes (see Table 3.1 for primer sequences). Each 25 μl reaction contained 3-5 μl of cDNA, 2.5 μl of 10X *Taq* DNA polymerase buffer B (Fisher Scientific, Pittsburgh, PA), 2.0 units of *Taq* DNA polymerase (Fisher Scientific, Pittsburgh, PA), 0.2 mM of each dexoynucleotide triphosphate, 1.5 mM magnesium chloride, 0.4 μM of each primer, 1 M betaine (Sigma-Aldrich, St. Louis, MO) and 5% dimethyl sulfoxide. PCR amplification was carried out under the following conditions: a single cycle at 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, 50-54°C for 1 minute, and 72°C for 1 minute, and a single cycle of 72°C for 10 minutes. Purified amplicons were sequenced and analyzed as described above.

Table 3.1. Primers and melting temperatures (°C) used for polymerase chain reaction amplification of canine *COL4A3* and *COL4A4*

Primer Set	Tm	Sequence	Primer Set	Tm	Sequence
COL4A3-1	56	GCTTTCCTGGACCTGCTG	COL4A4-1	57	GTCCCAAGACTTGACTGTGAGG
		GCGTCACAATAACTGTTCCTGG			GTCACCCTGAATACCTTTAATGGCA
COL4A3-2	54	GCCACAGAAGGTATAGAACTTGA	COL4A4-2	56	GGCTACAATGGTTCACGAGGT
		CAGCACTTCCAAGAACTCCAG			TGGTCCAATTCCAGGTTCTCC
COL4A3-3	53	GGTGAAGACGGCATTAAGG	COL4A4-3	56	GTACAGCCACCTGATTCCTGT
		CCTCAGGCTCAGATGTTTCTC			GGACTTAAATCCGCTCTCCCTG
COL4A3-4	54	CTCTTGTGCGTACAGTGTGC	COL4A4-4	57	ACTATGGAGAAATGGGGTCCGT
		GGATACCAGGTGGACCTC			GTTGTCCTGGAAATCCTGGGAG
COL4A3-5	52	CAAACTATGGACCACAGGG	COL4A4-5	56	ACCTGGCAGCAAAGGAGAA
		CTGGTTGCCCTTCCAATCCAT			CTCCCATGTCACCACGAAAAC
COL4A3-6	54	GGACTAGCCATACCTGGAGA	COL4A4-6	57	AGGGTTGAGGTGCTTGGATG
		GTCCTTTCTCTCCTTCTTCCC			CCTGGGACTCCTGGGAATC
COL4A3-7	55	GGTCATCGAGGTGAGATAGGA	COL4A4-7	57	AGGTTGGAAAGGACAGCGAG
		CTTGGAGACCATGAACACCTG			CTCTCAGCCCAGGTGAGC
COL4A3-8	56	CAGGCAGCATTGGGAATCC	COL4A4-8	57	AGCACCTGGATTTCACAGAGG
		AGTCCCGTCATGCCAGTG			AGCACCTTTGGCTCCTTTCTG
COL4A3-9	55	CTATGGGCATGAGAGGCAAC	COL4A4-9	56	AAGAGGCATGATGGGAGATCC
		GATCTTAACTGTGCCAGGGTCTC			CATCGCATCCTGGGAAGC
COL4A3-10	52	GTCTACCTGGAACTGTGG	COL4A4-10	57	GAGCGTTGACCTTCTGAGAGG
		CTGCCGAGAGTTCCCAAGTC			GAAGCCACTGAGGTATCCAGG
COL4A3-11	52	CCTGGATTAGATGGACTGC	COL4A4-11	56	CCCAAGAGGACCAGAAGGAG
		ATGAGGGAATGTCAGTGGT			TCTGAGAGCGGCATCATGG
COL4A3-12	54	GCACTGTCTGTGAAGGTCC	COL4A4-12	57	ACCAAGTGTGCCACTACGC
		GAAACTCTGCAAGAGCACAAG			ACTGGAGTCTGAAATGAGCACC

Tm, melting temperature

Genomic Screening of COL4A4 *Exon 30*

PCR amplification of exon 30 of *COL4A4* was performed as above for exon 3 using genomic DNA from the 2 affected ESS and 61 ESS of unknown status. Cycling conditions were as follows: 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, 54°C for 1 minute, and 72°C for 1 minute, and 1 cycle of 72°C for 10 minutes. PCR products were treated with 0.5 units of Exonuclease I and 0.25 units of shrimp alkaline phosphatase, and then sequenced and analyzed as described for exon 3 above.

Results

Clinical Findings

The 2 female ESS littermates (Dog 14 and Dog 15*) were owned by 1 individual who did not observe any signs of ill health before taking them to a veterinarian to be spayed at 7 months of age. Laboratory tests performed then showed that both dogs had marked proteinuria (Dog 14: USG, 1.036; UPC, 7.9, and Dog 15: USG, 1.042; UPC, 7.0). Both dogs were mildly hypoalbuminemic (Dog 14: SA, 2.2 g/dL, and Dog 15: SA, 2.5 g/dL; reference range, 2.7-4.4 g/dL). Dog 14 also was mildly azotemic (BUN, 48 mg/dL; reference range, 6-25 mg/dL; and SCR, 1.6 mg/dL; reference range, 0.5-1.6 mg/dL), and values for Dog 15 (BUN, 38 mg/dL; SCR, 1.3 mg/dL) were borderline high.

Additional testing performed 2 months later showed persistent proteinuria and progression to renal failure in both dogs, albeit more rapidly in Dog 14 (UPC, 9.8; BUN, 119 mg/dL; SCR, 6.2 mg/dL; and SA, 2.2 g/dL) than in Dog 15 (UPC, 8.1; BUN, 65 mg/dL; SCR, 3.0 mg/dL; SA, 2.2 g/dL). A renal ultrasound examination of Dog 14

*In keeping constant with the numbering of the dogs in Figure 5, the affected ESS are referred to as Dogs 14 and 15.

showed hyperechoic cortices with normal corticomedullary definition in both kidneys. Renal ultrasound findings for Dog 15 were similar except that the left kidney also exhibited a large wedge-shaped cortical defect that was thought likely to be the site of a previous infarct, and the right kidney was appreciably larger (about 1.5 cm longer) than the left kidney.

One week later, Dog 14 began exhibiting uremic signs and was brought to TAMU-CVM for euthanasia and postmortem examination of the kidneys. When examined, the dog was weak and moderately dehydrated. Laboratory tests performed on this occasion showed severe azotemia (BUN, 203 mg/dL; SCR, 11.3 mg/dL) and persistent proteinuria (USG, 1.021; UPC, 9.8).

Dog 15 remained clinically well for 1 additional month despite increasing azotemia (SCR concentration increased from 3.0 to 4.4 mg/dL over a 12-day interval) before the dog became lethargic and began vomiting. The dog then was brought to TAMU-CVM for euthanasia and postmortem examinations of the kidneys. The dog had a subdued demeanor and was marginally dehydrated when examined, and laboratory tests showed azotemia (BUN, 82 mg/dL; SCR, 6.2 mg/dL) and persistent proteinuria (USG, 1.016; UPC, 11.9).

Pathologic Findings

The gross appearance of both kidneys of Dog 14 was similar. They were symmetrically shaped and had smooth capsular contours. However, the kidneys of Dog 15 were not symmetrical; the left kidney was smaller than the right, and its middle third was smaller than its poles.

Histologic examination disclosed similar severe changes in the glomeruli and tubulointerstitium of both dogs. Glomeruli had thickened and often laminated Bowman's capsules. Glomerular tufts were lobulated and markedly hypercellular with widespread mesangial expansion and effacement of peripheral capillary loops. Hypercellularity within glomeruli consisted of increased numbers of mesangial cells coupled with hypertrophy of endothelia as well as visceral and parietal epithelial cells. Where present, patent glomerular capillary loops frequently were circumscribed by mildly to moderately thickened walls. Glomerular adhesions to Bowman's capsule (synechia) were common, and occasionally glomerular adhesions were accompanied by crescents of increased cells at the interface of the adherent glomerular tuft and the thickened capsule. Hyaline eosinophilic amorphous coagula and eosinophilic globular material were common within the urinary space. The cortical interstitium was diffusely and mildly to moderately expanded by dense to regionally loose (edematous) collagenous connective tissue containing widely scattered infiltrates of predominantly mixed mononuclear inflammatory cells. Cortical tubules often were mildly dilated and lined by mildly attenuated epithelia. Tubular dilatation also was regionally accentuated and often most prominent in distal segments. Eosinophilic proteinaceous fluid and protein casts were commonly present within tubular lumina and extended into the medulla. The medulla was diffusely and homogenously expanded by dense collagenous connective tissue containing widely scattered small foci of mixed mononuclear inflammatory cells.

Ultrastructural evaluation of the glomeruli identified diffuse effacement of visceral epithelial cell foot processes. Glomerular capillary walls exhibited multifocal

wrinkling and mesangial cell interpositioning. Basement membranes within the glomerular capillary walls were globally thickened due to distortion of the lamina densa, which was characterized by longitudinal splitting and fragmentation that created the appearance of a “net-like” or “woven” pattern (Figure 3.1A). Mesangium had increased cells and matrix.

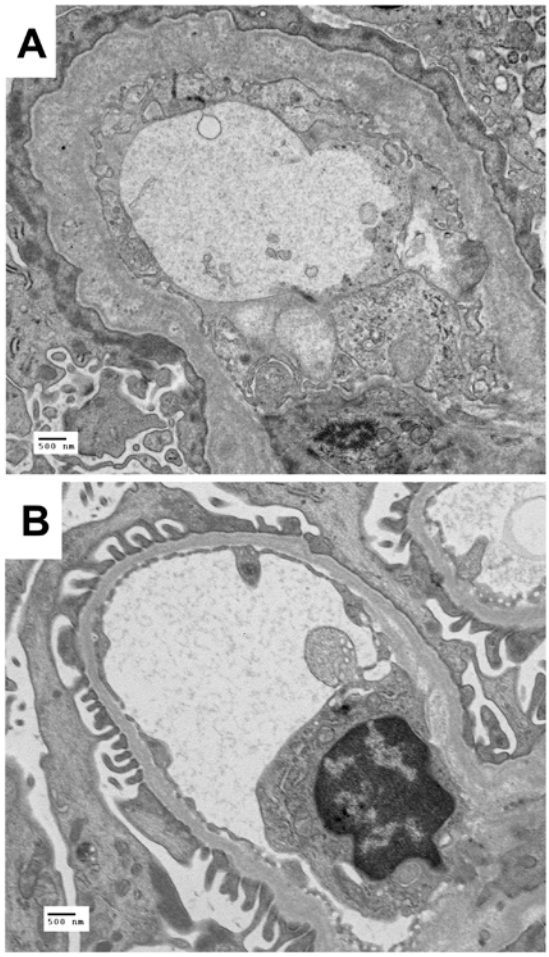

Figure 3.1 Transmission electron photomicrograph of a glomerular capillary loop from an ARHN-affected ESS (dog 15; panel A) that shows marked thickening of the glomerular basement membrane with longitudinal splitting and fragmentation of its lamina densa, as compared with a capillary loop from a normal dog (panel B).

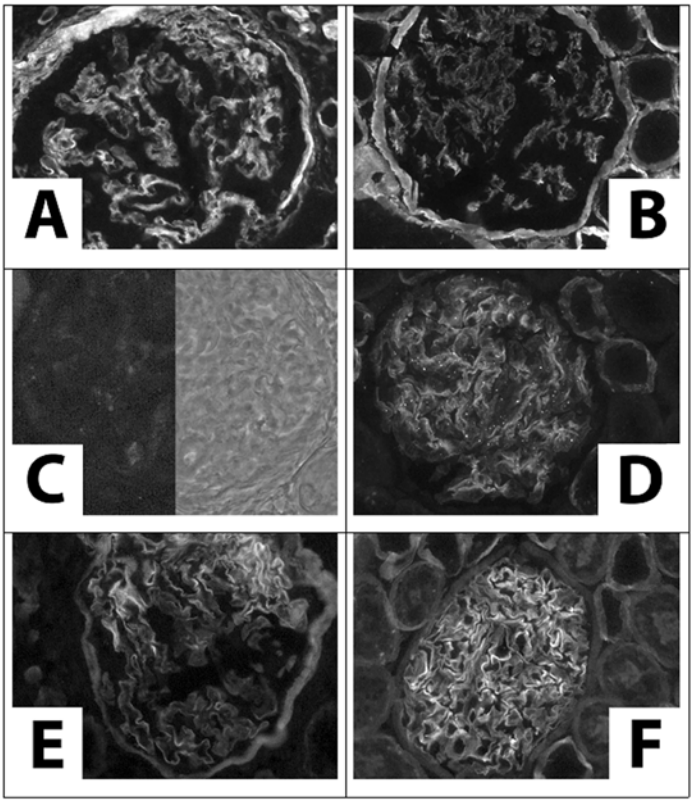

Figure 3.2 Fluorescence photomicrographs showing immunolabeling of renal sections from an ARHN-affected ESS (Dog 14; panels A, C, and E) compared with that for a normal dog (panels B, D, and F) for α1/2 chains (panels A and B), α3 chains (panels C and D), or α5 chains (panels E and F) of collagen IV. Panel A shows increased labeling of α1/2 chains, especially in the capillary walls, compared with normal dog kidney (Panel B). In panel C, which shows completely negative labeling for α3 chains in the ARHN-affected dog, half of the microscopic field is shown with phase-contrast illumination to verify presence of a glomerulus at that location. The negative labeling for α3 chains contrasts with the uniformly positive labeling of the normal dog's glomerular capillary walls for α3 chains (Panel D). Panel E shows labeling of the affected dog's capillary walls for α5 chains that is substantially reduced in its intensity and extent compared with the labeling of the normal dog's glomerular capillary walls for α5 chains (panel F).

Immunostaining of kidneys from both Dog 14 and Dog 15 produced similar results that demonstrated an abnormal pattern of expression of type IV collagens in their glomeruli (Figure 3.2). Labeling of GBM for α3 chains was completely absent (Figure 3.2C - D), whereas labeling for α5 chains was present but of decreased fluorescent intensity compared with normal dog kidney (Figure 3.2E - F) and labeling for α1/2 chains was increased (Figure 3.2A-B). This pattern of abnormal expression of type IV collagens in GBM was identical to that previously observed in ECS with ARHN (Davidson *et al*. 2007; Lees *et al*. 1998b).

Taken together, the pathologic findings were sufficient for an unequivocal diagnosis of ARHN in both affected ESS (Dog 14 and Dog 15) and identified *COL4A3* and *COL4A4* as candidate genes in which the underlying causative mutation was expected to exist in the dogs' kindred.

Genomic Screening of* COL4A4 *Exon 3

Exon 3 was sequenced and analyzed from the ARHN-affected ESS and compared to the sequences obtained from ECS of varying ARHN status to determine if this disease in ESS was caused by the same mutation identified in ECS. All sequences were aligned with the published genomic sequence (CanFam 2.0; (Lindblad-Toh *et al*. 2005)) for reference. Neither ARHN-affected ESS possessed the nonsense mutation found in affected and carrier ECS (data not shown).

Quantitative Reverse Transcription-PCR

Transcript quantities of *COL4A3, COL4A4 and COL4A5* mRNA were evaluated with qRT-PCR. Relative to unaffected dogs, quantities of *COL4A3* and *COL4A4* mRNA

transcripts were decreased 14.33-fold and 10.60-fold respectively, in the kidneys of ARHN-affected ESS. *COL4A5* mRNA numbers in kidneys of affected ESS were not significantly different from those seen in unaffected dogs (Figure 3.3).

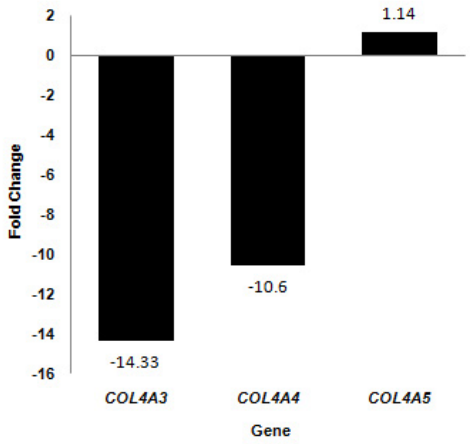

Figure 3.3 Quantitative reverse transcription PCR analysis of *COL4A3*, *COL4A4* and *COL4A5* mRNA transcript levels revealed a 14.33- and 10.6-fold decrease of *COL4A3* and *COL4A4*, respectively, in ARHN-affected ESS compared to mRNA levels in normal dogs. Transcript levels of *COL4A5* were not significantly different between the 2 groups.

COL4A3 and *COL4A4* cDNA Sequencing

To identify the causative mutation of ARHN in ESS, overlapping 500-bp segments spanning the coding regions of both *COL4A3* and *COL4A4* were sequenced. For comparison, cDNA from an unaffected, mixed-breed dog was sequenced with the same primer sets. All sequences were aligned with the published mRNA sequences for *COL4A3* (GenBank accession number AY263362.1) and *COL4A4* (GenBank accession number AY263363.1). A single nucleotide substitution was found at base 2806 in

COL4A4, changing a cytosine to a thymine in a region corresponding to exon 30. This substitution creates a nonsense mutation and is predicted to change the glutamine at position 904 to a stop codon, resulting in a prematurely truncated transcript (Figure 3.4). No other deleterious mutations were found in the coding sequences of either gene.

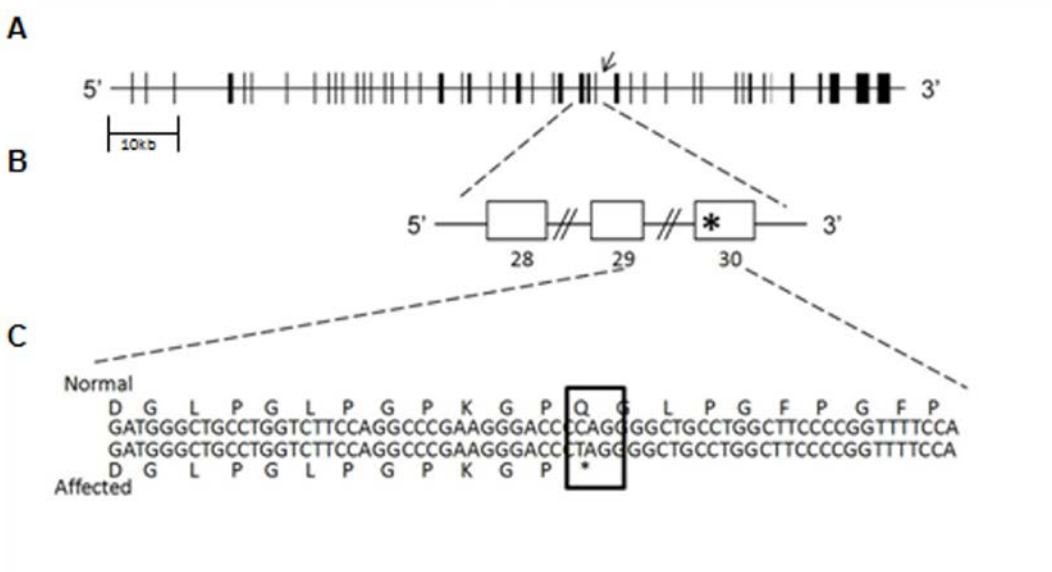

Figure 3.4 Diagram representing *COL4A4* including the mutation causing ARHN in the ESS. A is a model of the gene including the 47 exons and their introns. B is a magnification of the region encompassing exons 28, 29 and 30; the identified mutation is located in exon 30**.** C illustrates the predicted change to the protein sequence of the α4 (IV) collagen chain corresponding to the exon boundary between exons 29 and 30 (*Modified from Davidson et al., 2007).*

Genomic Screening of COL4A4 *Exon 30*

To confirm the mutation found in *COL4A4* cDNA, exon 30 was sequenced from the genomic DNA of 63 ESS. Both ARHN-affected ESS were homozygous for the

nonsense mutation (thymine). Thirteen of the 25 related ESS and 1 of the unrelated ESS were identified as carriers of the mutation, having both a cytosine and a thymine at base 2806. All other dogs screened were homozygous at this locus for cytosine (data not shown).

Discussion

This report documents the occurrence of ARHN in a family of ESS, a breed not previously reported to be afflicted with this disease. Affected ESS did not harbor the particular *COL4A4* mutation that is known to cause ARHN in ECS; therefore a different mutation in *COL4A4* or a mutation in *COL4A3* was anticipated. qRT-PCR was used to evaluate mRNA transcript quantities of the 2 candidate genes (*COL4A3* and *COL4A4*) as well as those of *COL4A5* in the kidney tissue of affected dogs with the intention of focusing the search for the mutation preferentially on 1 of the 2 genes. Because transcript numbers were similarly decreased for both *COL4A3* and *COL4A4*, sequencing of the coding regions proceeded for both genes. Sequencing of *COL4A3* cDNA did not reveal any mutations, but a nonsense C→T transition was found at base 2806, located in exon 30, of *COL4A4*. This point mutation changes a codon for a glutamine to a premature stop codon, and thus is predicted to produce a protein approximately half the normal length (904 of 1688 amino acids) and lacking the NC1 domain that is required for normal assembly of α3α4α5(IV) heterotrimers. The data strongly suggest this nucleotide change is the causative mutation that results in the disease state.

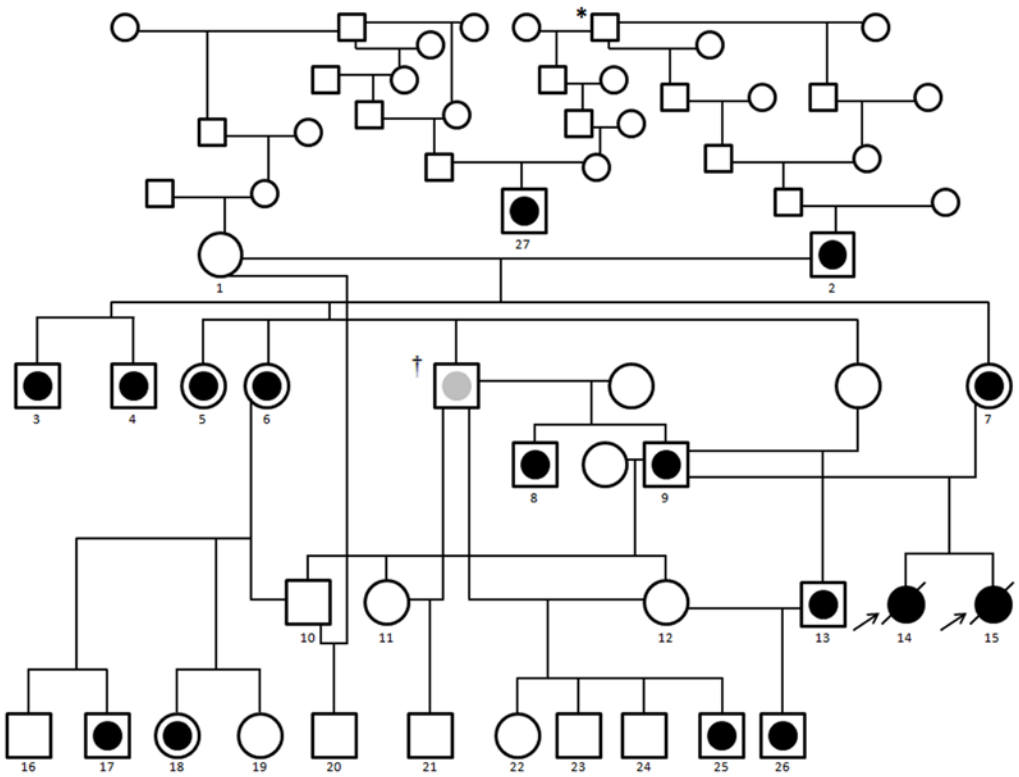

Figure 3.5 Pedigree of the related ESS. Squares represent male dogs; circles represent female dogs. Filled shapes indicate affected animals, dark circles within shapes indicate carrier status, and grey circles within shapes indicate presumed carrier status. Dogs 14 & 15 are the probands. Dog 27 is unrelated and shares a common ancestor (*****) with Dogs 1 & 2 4 or more generations back. The genotyping results from 1 related dog (†) were inconclusive; however, some of its progeny in multiple litters were carriers, and the dog thus is presumed to be a carrier. Only numbered dogs were included in the analysis.

Mutation screening for C2806T in related and unrelated ESS identified 13 carriers out of 25 related dogs (Figure 3.5) and 1 carrier among 35 unrelated dogs. Detailed

pedigree analysis identified a common ancestor for the unrelated carrier (dog 27) and the 2 founders of the family (dogs 1 and 2) 4 generations back. The common ancestor indicates that the mutation is not *de novo* within the nuclear family in Figure 5, and that additional carriers within the breed are likely. Given the small number of unrelated dogs tested in this study, it is not possible to predict the frequency of the mutant allele in the ESS population. Therefore, a population study to estimate the prevalence of carriers within the breed is warranted.

A genetic test for the mutant allele would be useful in confirming a diagnosis of suspected ARHN in an affected ESS. Such a test, however, would be most beneficial to ESS breeders in identifying carriers, thus aiding in breeding decisions to prevent the propagation of the disease. Because the prevalence of the mutant allele in the ESS population is unknown, all dogs in the breeding population might be tested to prevent the mating of 2 carriers and thus prevent further production of any affected dogs. Offspring from matings in which 1 parent is a carrier can be tested to select for dogs not carrying the mutation to be used as future breeding stock. In this way, the mutation can be removed from the ESS breeding lines in as few as 2 generations.

Finally, the findings in this report, together with the findings in previous reports, support several general statements about HN. To date, there are 4 different, fully characterized genetic forms of HN that have been identified. Two forms are X-linked, affecting Navasota mixed-breed dogs (Cox *et al*. 2003) and Samoyeds (Zheng *et al*. 1994), and each breed possesses a unique mutation in *COL4A5*. Two forms are autosomal recessive and are caused by distinct mutations in *COL4A4*, affecting ECS (Davidson *et al*.

2007) and ESS (described here). However, all 4 mutations cause disruptions of gene translation leading to the synthesis of truncated collagen IV alpha-chains, thus preventing the assembly of the α3α4α5(IV) network that is crucial for maintenance of GBM structure and function in the adult kidney. Therefore, the clinical disease produced by all 4 conditions is similar. Affected dogs appear completely healthy as puppies, developing persistent proteinuria of glomerular origin as their first manifestation of disease (Jansen *et al*. 1986; Lees *et al*. 1998a; Lees *et al*. 1999). The onset of proteinuria most often is at 4-6 months of age, but can be as early as 2-3 months or as late as 6-8 months of age. Progression of the disease thereafter is characterized by a period of increasing magnitude of proteinuria, followed by a period of gradually decreasing glomerular filtration rate and increasing azotemia, leading to end-stage renal disease, which most often occurs at about 12 months of age, but can occur as early as 6 months or as late as 24 months of age (Jansen *et al*. 1986; Lees *et al*. 1998a; Lees *et al*. 1998b; Lees *et al*. 1999). When dogs with juvenile-onset nephropathies are carefully evaluated, recognition of the clinical features of HN and exclusion of the clinical features of other conditions generally are sufficient to raise a high index of suspicion for the diagnosis of HN, but definitive diagnosis of the condition requires appropriate pathologic evaluations. Nevertheless, light microscopic examinations alone are not sufficient. The histologic lesions in the kidneys of HN-affected dogs are nonspecific changes that are indicative of glomerular disease but are not unique to HN. The distinctive morphologic feature of HN, which is the unique ultrastructural appearance of the GBM, can only be demonstrated by transmission electron microscopy. Notably, the distinctive ultrastructural GBM change is common to

all of the fully-characterized forms of HN that have been described, regardless of their mode of inheritance (Jansen *et al*. 1986; Lees *et al*. 1998a; Lees *et al*. 1999). The type of pathologic evaluation that can discriminate between the 2 genetic forms of HN is immunostaining that demonstrates the presence or absence of particular type IV collagen alpha-chains in the renal or epidermal basement membranes of affected dogs. The pattern of collagen IV chain expression observed in the ESS in this report is the same as that observed in ECS and is characteristic of ARHN (Lees *et al*. 1998b). There is no GBM labeling for α3(IV) chains, and GBM labeling for α5(IV) is decreased but not completely absent because α5(IV) chains combine with α6(IV) chains to form a network composed of α5α5α6(IV) heterotrimers in the GBM of ARHN-affected dogs. This contrasts with the complete absence of labeling for α3(IV), α4(IV), α5(IV), or α6(IV) chains in XLHN-affected male dogs (Harvey *et al*. 1998; Lees *et al*. 1999), which occurs because they cannot synthesize α5(IV) chains and therefore cannot assemble either α3α4α5(IV) or α5α5α6(IV) heterotrimers for expression in any basement membrane. Of note, despite the clinical and pathologic similarities that exist among these inherited nephropathies, each of the breeds or kindreds of dogs with fully-characterized HN that have been identified to date have had a different (*i.e*., unique to that breed or kindred) underlying causative mutation (Cox *et al*. 2003; Davidson *et al*. 2007; Zheng *et al*. 1994). The practical consequence of breed-specific mutations is the requirement of a different DNA-based genetic test in order to identify the mutant allele in each separate dog breed or kindred. Such an outcome is not surprising because several hundred different causative mutations have been identified in people with Alport syndrome, and nearly every affected family

worldwide has its own unique causative mutation in *COL4A3*, *COL4A4*, or *COL4A5* (Kashtan and Segal. 2011).

Acknowledgments

The authors gratefully acknowledge Mary Sanders and Kayla Alling for their technical assistance and the owners and breeders of the English Springer Spaniels who provided samples from the dogs that were used in this study.

CHAPTER IV

THE FUNCTION OF DOG MODELS IN DEVELOPING GENE THERAPY STRATEGIES FOR HUMAN HEALTH*

Overview

The domestic dog is of great benefit to humankind, not only through companionship and working activities cultivated through domestication and selective breeding, but also as a model for biomedical research. Many single-gene traits have been well-characterized at the genomic level, and recent advances in whole-genome association studies will allow for better understanding of complex, multigenic hereditary diseases. Additionally, the dog serves as an invaluable large animal model for assessment of novel therapeutic agents. Thus, the dog has filled a crucial step in the translation of basic research to new treatment regimens for various human diseases. Four well-characterized diseases in canine models are discussed as they relate to other animal model availability, novel therapeutic approach and extrapolation to human gene therapy trials.

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Introduction

Since sequencing of the canine genome was completed (Lindblad-Toh *et al.* 2005), the dog has been widely heralded for its utility as a model for human hereditary diseases (Galibert and Andre. 2006; Ostrander and Kruglyak. 2000; Ostrander and Wayne. 2005; Parker and Ostrander. 2005; Parker *et al*. 2010; Shearin and Ostrander. 2010; Spady and Ostrander. 2008; Sutter and Ostrander. 2004; Tsai *et al*. 2007) , owing in part to the natural occurrence of more than 250 hereditary diseases with very similar clinical presentations to analogous diseases in the human (Anonymous a). Dogs have been selectively bred, resulting in a range of phenotypes not observed in any other species. This is most notable in relation to size: the smallest breeds stand 15cm at the shoulder and weigh less than 3kg, whereas the largest breeds stand up to 110cm high and can weigh more than 45kg. More than 400 breeds of dog have been developed, each possessing unique behaviors and phenotypes. Each purebred dog is produced from a closed population: registered dogs must come from registered parents. However, dog varieties are freely able to interbreed, introducing diverse genetic backgrounds that can more accurately reflect genetic differences in human populations. Of additional interest to the many naturally-occurring diseases shared by human and dog and the phenotypic diversity among breeds is the emotional investment of owners with affected dogs; to this end, owners, veterinarians, and researchers alike have a strong commitment to developing preventive and therapeutic approaches to improve the health of pets.

Importantly, the comparable organ size between large breed dogs and the human makes the dog useful in clinical trials of new drugs, surgical procedures and gene

therapy. This latter issue is especially relevant because biological conditions relating to delivery of gene therapeutic agents can be more closely mimicked in a large animal model than in the mouse. Also, disease phenotypes in the mouse are not always identical to those in the human. For example, the mouse model of glycogen storage disease type Ia does not present with lactic acidosis, a hallmark symptom of the human disease (Kishnani *et al*. 1997). Another example is Duchenne muscular dystrophy, which, in the *mdx* mouse model does not show the characteristic muscle wasting seen in human DMD (Pastoret and Sebille. 1995). Most importantly, the immune response to viral vectors that occurs in mouse models can differ substantially from that observed in the human. These disparate immune responses necessitate additional trials to evaluate the virulence and immunoreactivity of vectors in additional model systems (Kishnani *et al*. 2001). Thus, dogs fill a critical void between mouse models and human trials for gene therapy.

Many disorders targeted for gene therapy studies are recessive. Therefore, small amounts of functional wild type product may presumably be sufficient to improve clinical signs. Preliminary studies in which canine cells were successfully transduced with retroviral vectors expressing transgenes illustrated the potential for the dog as a preclinical gene therapy model (Axelrod *et al*. 1990; Kwok *et al*. 1986). Shortly thereafter, a study using adenoviral vector delivery of Factor IX via the portal vein demonstrated initial success followed by a decrease in gene expression (Kay *et al*. 1994). Studies such as these helped establish a foundation for later work. Dogs are currently serving as models for gene therapy in many disorders, including hematological (Margaritis. 2010; Nichols *et al*. 2009), metabolic (Haskins *et al*. 2006; Koeberl *et al*. 2009), oncological (Hajitou. 2010),

ocular (reviewed in (den Hollander *et al*. 2010)), immunological (leukocyte adhesion deficiency [(Malech and Hickstein. 2007)], severe combined immunodeficiency [(Sokolic *et al*. 2008)]) and musculoskeletal (Duchenne muscular dystrophy [(Nakamura and Takeda. 2011)]) diseases (Table 4.1).

This review discusses four canine hereditary disorders: leukocyte adhesion deficiency, Duchenne muscular dystrophy, and two retinopathies (Leber's congenital amaurosis, and achromatopsia). Each is unique with respect to its reproducibility of human clinical signs, therapeutic approach, and extrapolation to human gene therapy trials.

Table 4.1 Human gene therapy trials based on preclinical trials in canine models

Disease	Initiation Date	Human Clinical Trial Number	Type of Study	Vector/Molecule Type
Gaucher's Disease[a]	1999	NCT00004294	Phase I	Retrovirus
Leukocyte adhesion deficiency[b]	2001	NCT00023010	Phase I Follow-up	Retrovirus
Hemophilia A[c]	2003[d]	No number provided	Phase I	Retrovirus
Duchenne Muscular Dystrophy[e]	2007	NCT00159250	Phase I/II	Antisense oligonucleotide
Hemophilia B[f]	2007	NCT00515710	Phase I	rAAV2
Leber Congenital Amaurosis, LCA2[g]	2007	NCT00516477	Phase I	rAAV2
Duchenne Muscular Dystrophy[h]	2009	NCT00844597	Phase I/II	Antisense oligonucleotide
Leber Congenital Amaurosis, LCA2[i]	2010	NCT01208389	Phase I/II	rAAV2
Leber Congenital Amaurosis, LCA2[j]	2011	NCT00999609	Phase III	rAAV2

[a] Novelli and Barranger 2001

[b] Bauer and Hickstein 2000

[c] Powell et al. 2003

[d] Year of publication

[e] Shimatsu et al. 2003, Kinali et al. 2009

[f] Xu et al. 2003, Manno et al. 2006

[g] Acland et al. 2001, Acland et al. 2005

[h] Shimatsu et al. 2003, Aartsma-Rus et al. 2004

[i] Maguire et al. 2008, Amado et al. 2010

[j] Acland et al. 2001, Maguire et al. 2008

Leukocyte Adhesion Deficiency-1

Leukocyte adhesion deficiency-1 (LAD-1) is a rare congenital autosomal recessive disease. The hallmark of LAD-1 is recurrent, potentially life-threatening bacterial infections (Anderson and Springer. 1987; Crowley *et al*. 1980). Other symptoms include progressive periodontal disease, persistent neutrophilia and defective wound healing, manifesting initially as delayed umbilical cord separation (Crowley *et al*. 1980). LAD-1 has a heterogeneous clinical presentation, with both a moderate and a severe form (Anderson and Springer. 1987). Those children affected by the severe form have a high incidence of death before the age of two years. With the moderate form, patients can live into young adulthood and, in rare cases, to middle age (Anderson and Springer. 1987).

LAD-1 is caused by a missense mutation in an integrin gene, *ITGB2*. This gene encodes integrin β subunit CD18 that, in normal cells, forms a cell surface adhesion complex with the α subunit CD11 (Kijas *et al*. 2000). This protein complex is critical to the adhesion function of leukocytes and plays a major role in inflammation and immunity (Arnaout. 1990). Defects in CD18 prevent formation of the CD11/CD18 complex and impair the ability of leukocytes to adhere to vascular surfaces (Anderson and Springer. 1987; Kijas *et al*. 2000). With the loss of adhesion, neutrophils are unable to extravasate from blood vessels and migrate to sites of infection and inflammation, leading to the chronic life-threatening bacterial infections that characterize this disease. Patient biopsies from infected tissues show low levels of granulocytes and a marked absence of neutrophils (Anderson and Springer. 1987; Weisman *et al*. 1985). Patients also show

especially high neutrophil counts, indicating that these cells are unable to leave the bloodstream and migrate to other tissues (Anderson and Springer. 1987).

Currently available treatments for LAD-1 are prophylactic antibiotic therapy and hematopoietic stem cell transplants (Bauer *et al*. 2004). Even with regular antibiotic therapy, patients develop infections that progressively worsen in the complete absence of neutrophils. Wound infections are very common and often require repeated surgical intervention to remove infected and necrotic tissue (Bauer *et al*. 2004). Allogeneic hematopoietic stem cell transplants have helped somewhat, but still have not been entirely successful at treating LAD-1 (Bauer *et al*. 2004). Children receiving these bone marrow grafts must undergo myeloablative radiation therapy before transplantation, which can cause lethal complications arising from infections or graft-versus-host disease (Thomas *et al*. 1995). The grafts are not always successful in treating the disease and may require a second graft. Interestingly, stem cell transplantation preceded by non-myeloablative conditioning has been shown to effectively treat other immunodeficiencies without the complications seen in myeloablative therapy (Bauer *et al*. 2004).

There are several animal models of LAD-1. The mouse model is a CD18 null mutant created to study CD18 function *in vivo* (Scharffetter-Kochanek *et al*. 1998). Although these mice exhibit clinical signs similar to human LAD-1, such as neutrophilia and neutrophil adhesion defects, the CD18 null mutant is not an ideal model on which to develop LAD-1 gene therapy. In contrast to mouse, bovine and canine leukocyte adhesion deficiencies (BLAD and CLAD, respectively) are spontaneously occurring diseases. BLAD-affected calves show similar clinical signs to human patients (e.g.,

defective wound healing, periodontitis, recurrent infections). Blood tests indicate that neutrophils from these calves have the same adhesion impairment and accumulate within blood vessels (Kehrli *et al*. 1992; Shuster *et al*. 1992; Shuster *et al*. 1992). The causative mutation in the bovine model is located within a region of the CD18 gene that is highly conserved across several species including human. BLAD is considered to be one of the most common genetic diseases in animal agriculture (Shuster *et al*. 1992). The annual economic loss in dairy production as a result of BLAD-affected calves has been estimated as high as $5,000,000 (Gerardi. 1996). With the persistence of the disease allele (Meydan *et al*. 2010; Patel *et al*. 2007; Powell *et al*. 1996; Wanner *et al*. 1998), BLAD continues to pose significant economic threat to cattle production worldwide.

CLAD is a model of the severe form of LAD-1 and has been described in juvenile Irish setters (Bauer *et al*. 2004). The signs of CLAD are identical to those seen in both bovine and human disease (Bauer *et al*. 2004; Giger *et al*. 1987; Renshaw *et al*. 1975; Trowald-Wigh *et al*. 1992). Neutrophils from affected dogs do not exhibit surface CD11/CD18 complexes and have the same adhesion deficiency as seen in human LAD-1 patients (Giger *et al*. 1987; Trowald-Wigh *et al*. 1992). The same therapies used for LAD-1 are used to treat CLAD, often with similar results. However, new therapies can be tested in CLAD for use with LAD-1 patients. The disease phenotype was successfully reversed in afflicted pups using a non-myeloablative conditioning regimen prior to stem cell transplantation, leading to the recommendation of this treatment for human LAD-1 patients (Creevy *et al*. 2003). Although hematopoietic stem cell transplantation methods

are improving, the treatment does not have a high efficacy and is still prone to complications.

This led to the development of gene therapy approaches. A gene therapy study involving two human LAD-1 patients was recently completed in the United States. A retroviral vector containing CD18 had been administered to the patients during a Phase I clinical trial in 1999 (FDA investigational new drug study BB-IND-7949), and the follow-up study was intended to monitor the long-term outcome of the therapy although results have not yet been released (reviewed in (Bauer and Hickstein. 2000); ClinicalTrials.gov number NCT00023010). Since this human clinical trial took place, there have been several *ex vivo* gene therapy studies assessing efficacy of different viral vectors in the dog model (Bauer *et al*. 2008; Bauer *et al*. 2011; Hunter *et al*. 2011; Nelson *et al*. 2010). In *ex vivo* therapy, target cells are removed from the subject and transduced with a viral vector containing a transgene. The subject undergoes a conditioning treatment of total body irradiation and receives an infusion of the autologous transduced cells. In the case of LAD-1 *ex vivo* gene therapy, bone marrow is removed from subjects and hematopoietic progenitor cells are isolated by flow cytometry for transduction and subsequent infusion.

Studies using lentiviral (LV) and foamy virus vectors containing human elongation factor 1α and PGK promoters, respectively, to drive expression of CD18 were unable to reverse the disease phenotype in CLAD dogs (Bauer *et al*. 2011; Nelson *et al.* 2010). A recently published study showed that using a specific region of either the human CD11b or CD18 promoters to drive canine CD18 expression in a self-inactivating (SIN)

LV vector resulted in CD18 expression on neutrophils. This was sufficient to reverse CLAD in three of the four dogs receiving gene therapy (Hunter *et al*. 2011). Use of the SIN LV vector is thought to reduce the risk of genotoxicity, making this construct a potential candidate for gene therapy treatment of patients with LAD-1 (Zufferey *et al*. 1998).

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a progressive degenerative disorder with an X-linked recessive pattern of transmission. It is the most common and most severe form of muscular dystrophy (Emery. 2002). The prevalence is approximately 1 in 3500 in males (Koenig *et al*. 1988; van Essen *et al*. 1997). Characteristic symptoms of DMD include impaired walking and running, muscle weakness, contractures, enlargement of the calf muscles due to infiltration of adipose tissue, and skeletal muscle degeneration leading to confinement to a wheelchair before puberty for most patients. Serum creatine kinase (CK) levels are increased as a by-product of muscle breakdown. Intellectual disability (IQ < 70) is seen in 20% of affected males (DMD; OMIM #310200). It was common for death to occur before the age of 25 due to pneumonia with cardiac involvement; however, as advances in respiratory care are made, patient survival has increased into the early 30s (Emery. 2002).

DMD is caused by a lack of dystrophin, which is thought to be involved in cytoskeletal stability (Dalkilic and Kunkel. 2003; Hoffman *et al*. 1987). Dystrophin, encoded by *DMD*, localizes to the muscle cell membrane and binds other membrane proteins at its C-terminus to form the dystrophin-glycoprotein complex. At the N-terminus, it binds actin and the cytoskeleton (Campbell. 1995; Oshima *et al*. 2009). Deleterious mutations in

DMD cause a loss of dystrophin, and thus instability of the glycoprotein complex and deterioration of the sarcolemma (Dalkilic and Kunkel. 2003). Between 60-65% of DMD patients carry a deletion of one or more exons of the *DMD* gene, most frequently between exons 45 and 52. Other identified causes of DMD include small insertions or deletions, point mutations and duplications in the gene (Oshima *et al*. 2009).

There are no pharmacological agents that effectively manage symptoms. Despite various reports of glucocorticoid therapies having a short-term slowing effect on progress of the disease, none of the trials used universal treatment regimens or the same drugs; thus, no consensus on the long-term effects of treatment has been reached (Emery. 1993). Surgical intervention is not recommended due to risks from anesthesia (e.g., malignant hyperthermia) and detrimental effects from bed rest following surgical procedures. No significant improvement in muscle strength or ambulatory ability is conferred by prophylactic surgery; however, patients do benefit from surgical correction just prior to or at the occurrence of contractures (Emery. 1993; Goertzen *et al*. 1995).

Several animal models of DMD exist, including the mouse and dog. A spontaneously occurring phenotype, the *mdx* mouse, was described in an inbred colony of C57Bl/10 mice characterized by increased serum CK levels and muscle lesions similar to those seen in DMD patients (Bulfield *et al*. 1984). The causative mutation in the *mdx* mouse was identified as a single base transition in the mouse *dystrophin* gene causing a dystrophin deficiency in all but ~1% of skeletal muscle fibers (Sicinski *et al*. 1989). The *mdx* mouse undergoes a continuous cycle of muscle degeneration and regeneration for most of its life, which is in contrast to human DMD patients whose muscles are replaced

with accumulations of adipose and connective tissue as they necrose. It is only in aging *mdx* mice that regeneration is impaired and adipose accumulations begin (Pastoret and Sebille. 1995). A second mouse model is the utrophin/dystrophin double knockout (dKO) mouse. These mice show similar signs of myopathy to *mdx* mice but the phenotype is more severe, including cardiomyopathy (Grady *et al*. 1997; Yue *et al*. 2006). Although the dKO mouse more closely resembles DMD, both models are still limited by the size of the mice and the potential for differential immune reactions to viral vectors.

Golden retrievers, a large breed dog, are affected by an X-linked severe progressive myopathy called Golden Retriever Muscular Dystrophy (GRMD). Affected dogs carry a mutation which causes faulty RNA processing of *dystrophin*, skipping exon 7 and transcribing exon 8 out of frame (Sharp *et al*. 1992). Characteristic signs of the disease are gait abnormalities, gradual skeletal muscle atrophy and increased serum CK levels (Cooper *et al*. 1988; Kornegay *et al*. 1988). Muscle contractures cause limb deformities, and much like DMD, respiratory failure and cardiomyopathy are common (Nguyen *et al*. 2002; Sharp *et al*. 1992). Distinctive signs of GRMD (and both canine models discussed below) are macroglossia and dysphagia (Kornegay *et al*. 1988; Shimatsu *et al*. 2003; Walmsley *et al*. 2010). The skeletal and cardiac signs of GRMD more closely resemble DMD than the *mdx* model. This, along with the larger body size of the golden retriever compared to the mouse, makes it a compelling animal model in the study of DMD (Nakamura and Takeda. 2011).

There are two additional canine models of DMD. The canine X-linked muscular dystrophy in Japan ($CXMD_J$) model was developed in the beagle, a medium-sized breed,

from a GRMD-affected golden retriever and carries the same mutation. Briefly, an unaffected female beagle was crossed with a male GRMD-affected golden retriever. Female offspring from this mating carrying the mutation were mated with normal male beagles to establish a breeding colony (Shimatsu *et al*. 2003). This model manifests the same clinical signs in a milder phenotype than the GRMD (Shimatsu *et al*. 2003). Recently, these signs were described in a toy breed, the Cavalier King Charles Spaniel (CKCS-MD). This breed presents with a severe spontaneous phenotype analogous to human DMD caused by a deletion of exon 50 of the dystrophin gene. Deletions in this exon are the most common causative mutations in human patients (Walmsley *et al*. 2010).

Gene therapy studies have been carried out in mouse and dog models of DMD using a shortened functional form of dystrophin which can be incorporated into recombinant adeno-associated viral (rAAV) vectors. Expression of both micro- and mini-dystrophin has been demonstrated to recover muscle function in the *mdx* and dKO mouse models (Gregorevic *et al*. 2008; Wang *et al*. 2009; Watchko *et al*. 2002; Yoshimura *et al*. 2004). In a recent study, both *ex vivo* gene therapy and electrotransfer of the plasmid encoding the microdystrophin were used to successfully express the transgene in both the *mdx* mouse model and the GRMD dog model (Pichavant *et al*. 2010). After administration of a rAAV9 human mini-dystrophin vector to neonatal dogs lacking dystrophin, expression of the construct was seen throughout the skeletal muscles. However, an immune reaction to the treatment was also noted, possibly due to the promoter or a human protein in canine muscle (Kornegay *et al*. 2010). Intravenous injection of a rAAV8 microdystrophin to

$CXMD_J$ dogs was able to induce widespread expression of the transgene for the eight-week duration of the study (Ohshima *et al*. 2008).

Exon-skipping therapy using antisense oligonucleotides (AO) is another form of gene therapy being studied for DMD (Kinali *et al*. 2009; Saito *et al*. 2010). As discussed above, many patients have a mutation in a splice site which, during transcription, causes deletion of an exon and a frameshift mutation (Oshima *et al*. 2009). The goal of exon skipping therapy is to recover a shortened functional protein from a gene harboring a mutation. By binding AO to the region containing the mutation, the ribosome is able to bypass the bound region and resume transcription further down the gene (van Deutekom. 2005).

Successful expression of functional dystrophin following skipping of exon 23 has been shown in the *mdx* mouse (Fletcher *et al*. 2006; Jearawiriyapaisarn *et al*. 2008; Lu *et al*. 2005). Recent work demonstrates that a multiple-exon skipping technique (exons 6-8) using injection of morpholinos, a type of AO, results in widespread expression of functional shortened dystrophin in the $CXMD_J$ model (Yokota *et al*. 2009). Using this same technique *in vitro*, similar dystrophin recovery was achieved in fibroblasts harvested from a human DMD patient with an exon 7 deletion (Saito *et al*. 2010). AO-mediated skipping of exon 51 in the CKCS-MD model successfully restored dystrophin expression in skeletal muscles. The CKCS-MD will be a valuable contribution to preclinical trials for DMD as the majority of human deletions are between exons 45 and 52 (Aartsma-Rus *et al*. 2004; Walmsley *et al*. 2010). Phase I/II clinical trials for this therapy

in human patients are currently underway in the UK ((Kinali *et al*. 2009); ClinicalTrials.gov NCT00844597).

Leber's Congenital Amaurosis

Leber's congenital amaurosis (LCA) is a group of autosomal recessive degenerative retinopathies causing bilateral partial or total blindness in infancy (LCA; OMIM #204000). Characteristic symptoms of LCA are nystagmus, eye poking behavior and severe visual impairment with little or no light perception. Signals from electroretinogram (ERG) testing are undetectable (Perrault *et al*. 1999). Although the photoreceptors lining the fundus appear normal initially, an abnormal appearance (e.g., salt-and-pepper pigmentation, atrophy of the retinal pigment epithelium) progressively develops over several years. LCA has a prevalence of approximately 1/81,000 (Stone. 2007).

There are 14 subtypes of LCA, designated LCA1-LCA14, which are differentiated by the genes harboring the causative mutations (LCA; OMIM #204000). This review will focus on LCA2, caused by mutations in *RPE65*, as this subtype is one of the most well-understood (Cai *et al*. 2009; den Hollander *et al*. 2008; Marlhens *et al*. 1997). Marlhens and colleagues identified two distinct mutations in *RPE65* in two siblings with LCA2. The patients were compound heterozygotes for both mutations, inheriting one from each parent, causing two distinct truncated proteins (Marlhens *et al*. 1997). The gene product, RPE65, localizes to the retinal pigment epithelium and is involved in regeneration of visual pigment in photoreceptors. Defective or absent RPE65 impairs phototransduction and can cause partial or complete blindness (Nicoletti *et al*. 1995).

The briard, a large breed dog, is afflicted by an inherited congenital retinal dystrophy caused by a four nucleotide deletion in *RPE65* (Veske *et al*. 1999). The causative mutation in briards is not the same mutation found in humans affected by LCA. However, both canine and human mutations result in a complete loss of RPE65, and the briard is a canine model of LCA2 (Acland *et al*. 2001). Similar to human patients, briards have night blindness, various degrees of impaired vision in daylight and undetectable or abnormal ERG readings (Narfstrom *et al*. 1989; Veske *et al*. 1999). Nystagmus is present in affected puppies; although the dogs outgrow this aspect of the disorder, excitement can induce a recurrence (Aguirre *et al*. 1998). Signs of the disorder were initially thought to be static, but close observation of affected dogs revealed a progressive component to the disease (Aguirre *et al*. 1998; Narfstrom *et al*. 1989).

A *RPE65-/-* mouse was created during the same time period the briard was initially characterized. While the mouse model is of importance to understanding the function of RPE65 *in vivo*, there are clear differences between the phenotypes of the *RPE65-/-* mice and human LCA2 patients. Human patients have rod and cone dysfunction, whereas the knockout mice retain functional cones (Redmond *et al*. 1998). Because of this difference, *RPE65*-deficient mice are not the optimal system in which to validate new therapies for LCA2.

In 2001, a proof-of-concept gene therapy study was performed in affected briards. Recombinant AAV vector expressing wild-type RPE65 delivered intra-ocularly to *RPE65*$^{-/-}$ dogs was able to restore vision. In each dog, the vector was delivered subretinally to only one eye (Acland *et al*. 2001). A Phase I/II clinical trial delivering the

same vector construct unilaterally to three human LCA2 patients soon followed (ClinicalTrials.gov number NCT00516477). This treatment resulted in improvements in visual acuity and pupillary reflex that lasted at least the six months of follow-up during the study (Maguire *et al*. 2008). Based on these preliminary results, a Phase III clinical trial was scheduled to begin in January 2011 with 12 patients (ClinicalTrials.gov number NCT00999609).

Because both of these studies had only examined viral delivery to one eye, a recent study investigated the safety of subretinal readministration of rAAV vectors to the same animal in the contralateral eye (Amado *et al*. 2010). RPE65-containing AAV was administered to the contralateral eye of *RPE65*$^{-/-}$ dogs previously treated with the vector constructs to determine the safety and efficacy of a second treatment. Not only did the gene therapy restore vision to the treated eye, but no adverse reactions were seen as a consequence of a second administration of the viral vector (Amado *et al*. 2010). Phase I/II clinical trials to examine the safety and efficacy of readministration to the contralateral eye in human patients are currently ongoing (ClinicalTrials.gov number NCT01208389).

Achromatopsia

Another form of hereditary retinopathy affecting dogs and humans is achromatopsia. The term achromatopsia can refer to both complete and incomplete forms of the disorder, as well as acquired and congenital forms. This review addresses complete congenital achromatopsia, an autosomal recessive cone dysfunction sometimes referred to as rod monochromacy (Michaelides *et al*. 2004). Approximately 1 in 30,000 people are affected, with initial diagnosis made during infancy. Symptoms include photophobia,

impaired visual acuity and nystagmus. Electroretinogram (ERG) readings indicate an absence of cone function yet normal rod function. Nystagmus, and in some cases, acuity, improve during development; however, color vision is lacking in human patients (Michaelides *et al*. 2004; Michaelides *et al*. 2006). The disorder does not appear to have a progressive component, remaining stable throughout the patients' lives.

Achromatopsia is genetically heterogeneous; mutations in four distinct genes involved in the cone phototransduction cascade are associated with the disorder. *GNAT2* encodes the α subunit of cone transducin (Gropp *et al*. 1996). Mutations in this gene are considered to be 'leaky' and cause a less severe phenotype because a small amount of correctly spliced mRNA transcripts are produced (Rosenberg *et al*. 2004). Mutations in the genes encoding either the α subunit of cone phosphodiesterase, *PDE6C* (Thiadens *et al*. 2009), or the α and β subunits of the cone photoreceptor-specific cyclic nucleotide-gated (CNG) channel, *CNGA3* and *CNGB3* respectively, cause the full phenotype of complete achromatopsia (Ding *et al*. 2009; Kohl *et al*. 2000; Milunsky *et al*. 1999; Wissinger *et al*. 1998; Wiszniewski *et al*. 2007).

The CNG channel is localized to the cone photoreceptor plasma membrane. In darkness, the channel is open and the cells are in a state of constant depolarization. Light activates the phototransduction cascade, causing the channel to close and hyperpolarize the photoreceptor cell. The subunits of the channel form heterotetramers (two α subunits and two β subunits) and are thought to be functionally distinct: the α subunit conducts ionic activity, and the β subunit functions as a modulator (Gerstner *et al*. 2000). More than 50% of achromatopsia cases are caused by mutations in *CNGB3*. Thr383fsx is the most

common mutation, accounting for over 70% of mutant *CNGB3* alleles. The frameshift caused by this mutation prematurely truncates the protein; thus, no functional protein is produced, preventing the formation of CNGA3/CNGB3 heterotetramers (Kohl *et al*. 2005; Wiszniewski *et al*. 2007).

Canine cone degeneration (*cd*) has been established as a valid animal model of achromatopsia caused by *CNGB3* mutations. Two separate phenotypes have been identified in the German Shorthaired Pointer (GSP) and the Alaskan Malamute-derived (AMD); two distinct mutations segregate with each phenotype. GSPs experience day blindness caused by a missense mutation in *CNGB3.* A deletion of at least 140 kb in the same gene causes *cd* in the AMD lineage (Sidjanin *et al*. 2002). Affected dogs have clinical signs very much like those seen in the human: impaired visual acuity, photophobia and nystagmus beginning around 8 to 12 weeks of age (Aguirre and Rubin. 1974; Rubin *et al*. 1967). Although cone-specific ERG signals are detectable at three to six weeks of age, adult dog retinas completely lack cone function and ERG signals are undetectable (Aguirre and Rubin. 1975).

Gene therapy for achromatopsia is being developed for the dog *cd* models (Komaromy *et al*. 2008; Komaromy *et al*. 2010). Initial work to determine the optimal promoter to drive gene expression of a green fluorescent protein (GFP) in a recombinant AAV5 vector showed that the red cone opsin promoter confers a higher level of specificity and greater expression levels than the human S-opsin promoter (Komaromy *et al*. 2008). Subsequent studies showed that unilateral subretinal injection of human *CNGB3* cDNA driven by the red-cone opsin promoter in a rAAV5 vector was able to restore cone

function in the treated eye in both phenotypes. Rescue of function was measured by both cone-specific ERG and vision behavior under bright light conditions. Because *CNGB3* mutations are the predominant cause of achromatopsia, this treatment could also offer a promising therapy to human patients (Komaromy *et al*. 2010).

Summary

Characterization of dog models of disease has provided a strong foundation for understanding the functional significance of hereditary disease. In some cases, dog models have preceded mouse models and in others, dog models more accurately reflect the human disease progression, clinical signs, and response to treatment than the mouse. While only a handful of diseases are being actively investigated in advanced (phase III/IV) human gene therapy trials, (e.g., Leber's congenital amaurosis), recent studies in the dog offer great promise of potential therapies for lysosomal storage diseases, including glycogen storage disease Ia (Koeberl *et al*. 2008; Weinstein *et al*. 2010), Hurler syndrome (Ellinwood *et al*. 2011; Traas *et al*. 2007), Sly syndrome (Mango *et al*. 2004; Wang *et al*. 2006; Xu *et al*. 2002), and Sanfilippo syndrome (Ellinwood *et al*. 2011); coagulopathies, including hemophilia A and B (reviewed in (Margaritis. 2010)); retinopathies such as LCA6 (Lheriteau *et al*. 2009); and cancers, including astrocytoma (Pluhar *et al*. 2010), sarcoma (Finocchiaro *et al*. 2011), and general neoplasms (Hajitou. 2010; Reed *et al*. 2010; Thamm *et al*. 2010).

By discussing the role of the dog in gene therapy trials, we hope to highlight the unique attributes of the dog, not only as a model for understanding simple traits, but also as a critical member of the therapy development pipeline for human health. While the

importance of mouse models cannot be overstated, data being generated from current, basic research in additional model organisms will enhance the knowledge base for translational medicine to draw upon for future therapies.

CHAPTER V

SUMMARY

The 7.5x canine genome assembly was, and is, an indispensable resource for study of canine genetics. However, despite substantial work by a number of laboratories to improve the reference genome, gaps in coverage contain 1% of the euchromatic genome. Such regions likely contain GC-rich promoter regions as well as repetitive sequences. Amplification and sequencing through promoters and 5′ regions of genes is very difficult because regions with high GC content form secondary structures that stall DNA polymerase.

A gap in CFA 25 between *COL4A3* and *COL4A4* has prevented identification of the full sequences of *COL4A3* and the predicted promoter. Mutations in each of these genes are known to cause HN. Using degenerate PCR primers, we were able to amplify and sequence 39 bases of exon 1 and the 32 bases of exon 2 of *COL4A3*. Determining the sequence of the promoter and 5′ regions of *COL4A3* and *COL4A4* contributes to coverage of the canine genome, and may be critical for identifying novel mutations associated with HN.

Although gaps in sequence coverage only encompass a small percentage of the canine genome, efforts by our lab and others focused on filling in these regions are necessary. As revealed by this work, gaps may harbor promoters and genes associated with severe disease phenotypes (*e.g.*, autosomal dominant HN). There exists an urgent

need to identify the genes and/or regulatory elements contained within these regions to uncover novel mutations causative for such diseases.

Recently a 50x coverage assembly of the collie genome was generated by our laboratory using the Illumina next generation sequencing platform. According to our assembly parameters, only paired-end reads aligning at both ends to the canine reference genome sequence were incorporated into the assembly. Discordant reads were not included; however, there may be a number of discordant reads for which one end may align to sequences flanking the gap regions. These may be used to carry out chromosome walking in order to span the gap. Even if this provides only single coverage of the gap region, the resulting sequence will still be very useful (Kirkness *et al*. 2003). If this successfully bridges the gap located on CFA25 between *COL4A3* and *COL4A4*, chromosome walking may be equally useful in sequencing through other regions missing from the canine reference genome.

Little is known about mechanism by which type IV collagen gene bifunctional promoters drive expression of gene pairs, and access to the full sequence of the promoter and both genes will allow for cloning and functional characterization of the promoter. Identification of binding sites within the promoter as well as promoter-proximal elements found in either gene could provide information about type IV collagen biochemical pathways and mechanisms of individual or tandem gene expression. Recognition and characterization of promoter-proximal elements could potentially assist in our understanding of the variability in disease severity observed in patients with AS resulting from different mutations.

HN is a devastating disease affecting several breeds of dog. Dogs affected by X-linked and autosomal recessive HN usually reach ESRD by 12 months of age. We identified a novel *COL4A4* mutation that segregates with the disease phenotype in a family of ARHN-affected ESS. Prior to diagnosis of ARHN in the probands, the disease was not previously known to affect the ESS. As with other traits inherited through autosomal recessive transmission, it is difficult to identify carriers until affected offspring are produced. The symptoms of HN do not appear until ESRD and are identical to those seen in other forms of acute renal disease. It is impossible to diagnose HN without confirmation by immunostaining and transmission electron microscopy of renal biopsy samples. Therefore, the disease may not be correctly diagnosed in dogs presenting with signs of ESRD.

The disease allele was identified by a simple test based on PCR amplification and sequencing of the exon containing the mutation. The screening test will be highly beneficial because it will identify dogs that carry the deleterious allele and such dogs can be removed from breeding lines. Through increased awareness of HN in the ESS by the veterinary community, along with identification of carriers and careful breeding practices, the mutation can be eradicated from the breed in as few as three generations.

APPENDICES

Appendix A

Methods for Amplifying and Sequencing GC-Rich Templates

Efforts to sequence across the gap on CFA 25 between *COL4A3* and *COL4A4* required many modifications to amplification protocols and cycling parameters. In order to assist future endeavors to sequence GC-rich regions, the following table provides names and suppliers of the reagents used as well as the final concentration or ranges of concentration of the reagents in each reaction.

Table A-1. Reagents used to optimize PCR for amplification of GC-rich templates

Reagent Name	Supplier	Concentration
PCR additives		
Betaine	Sigma-Aldrich	0.5-2.0 M
Dimethyl sulfoxide (DMSO)	Fisher Scientific	5-11%
7-deaza-dGTP	New England Biolabs	50 μM
Bovine serum albumin (BSA)	New England Biolabs	400 μg/ml
Magnesium source		
Magnesium chloride ($MgCl_2$)	Fisher Scientific	1.0-4.0 mM
Magnesium sulfate ($MgSO_4$)	Invitrogen	4.0 mM
Polymerases		
Taq DNA polymerase	Fisher Scientific	1.25-2.0 U
Advantage2 GC polymerase	Clontech	1X
Phire HotStart II polymerase	Finnzymes	1 μl

Appendix B

A missense mutation in the 20s proteasome β2 subunit of great danes having harlequin coat patterning*

Overview

Harlequin is a pigmentary trait of the domestic dog that is controlled by two autosomal loci: the melanosomal gene, *SILV*, and a modifier gene, *harlequin* (*H*), previously localized to chromosome 9. Heterozygosity for both a retrotransposon insertion in *SILV* and a mutation in *H* causes a pattern of black patches on a white background. Homozygosity for *H* is embryonic lethal. Fine mapping of the *harlequin* locus revealed a 25 kb interval wherein all harlequin Great Danes are heterozygous for a common haplotype. This region contains one gene, *PSMB7*, which encodes the β2 catalytic subunit of the proteasome. Sequence analysis identified a coding variant in exon 2 that segregates with harlequin patterning. The substitution predicts the replacement of a highly conserved valine with a glycine. Described herein is the identification of a naturally-occurring mutation of the ubiquitin proteasome system that is associated with a discernable phenotype of dogs.

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1. Introduction

The coat of the domestic dog is diverse in color, pattern, length, and texture. To date, at least 10 genes governing canine coat variation have been identified (Cadieu *et al*. 2009; Housley and Venta. 2006; Schmutz and Berryere. 2007), but many distinguishing traits have yet to be described at the genetic level. Harlequin is a coat pattern of distinctive dark spots on a white background (Fig. B-1A). Although most coat variation in dogs is controlled by a single locus, harlequin is a genetically complex coat pattern, resulting from heterozygous mutations at two loci: *merle* (*M*) and *harlequin* (*H*) (O'Sullivan and Robinson. 1988; Sponenberg. 1985). By itself, the *Mm* genotype causes a more colorful pattern, termed merle, characterized by dark spots on a dilute background (Fig. B-1B). *H* is a dominant modifier of *M* that removes the dilute pigment and increases the size of the fully-pigmented regions. *H* has no visible phenotypic effect in *mm* (wild-type) dogs (Fig. B-1C); therefore, merle is the only phenotype that unequivocally indicates an *hh* genotype. The base coat color of harlequin and merle dogs is controlled by separate loci (Schmutz and Berryere. 2007).

We previously determined that a retrotransposon insertion near an exon boundary of the melanosomal gene *SILV* (also known as *Pmel17*) is responsible for the merle coat pattern (Clark *et al*. 2006). *SILV* encodes the fibrillar array on which melanin is deposited in eumelanosomes and is strongly expressed during embryonic development (Baxter and Pavan. 2003; Theos *et al*. 2005). A cryptic splice acceptor site within the insertion causes retrotransposon sequence to be incorporated into *SILV* mRNA in merle dogs(Clark *et al*. 2008).Variability in the number of di-nucleotide repeats within the insertion results in

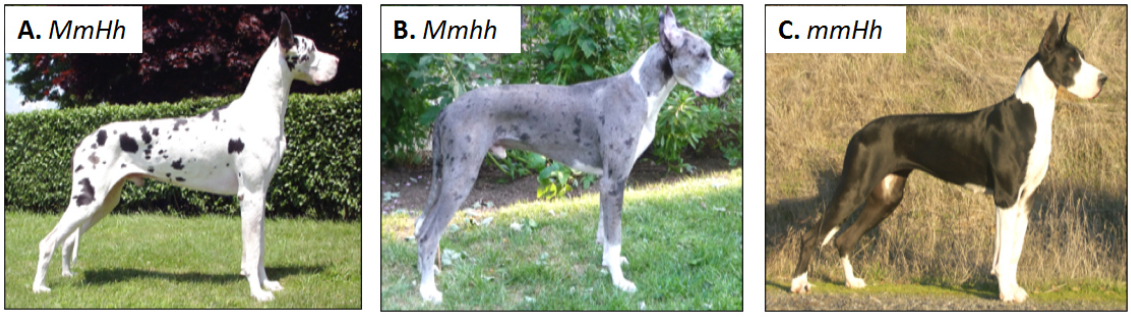

Figure B-1. Phenotypes resulting from genetic variations at the *M* and *H* loci in the Great Dane. (A) A classic harlequin with black patches on a white background. (B) A merle, characterized by black patches on a dilute background. (C) A non-merle harlequin, phenotypically indistinguishable from *mmhh*.

multiple mutant *SILV* transcripts (Clark *et al*. 2008). Truncation of the poly-A tail of the retrotransposon permits proper splicing and normal pigmentation (Clark *et al*. 2006). The random dark spots characteristic of the merle and harlequin patterns may be the result of somatic replication errors during development that truncate the poly-A tail (Clark *et al*. 2008).

Harlequin is most common in the Great Dane breed, although reports of the pattern appearing spontaneously in merle populations of other breeds are not uncommon. Comprehensive studies of Great Dane breeding records show that harlequins do not breed true, indicating that they are heterozygous for *H*, and that the *HH* genotype is not viable (O'Sullivan and Robinson. 1988; Sponenberg. 1985). Because harlequin-to-harlequin matings are not associated with stillbirths, *HH* likely causes lethality early in embryogenesis. In order to map the *H* locus, six small, multigenerational pedigrees of harlequin and merle Great Danes were previously assembled for use in a genome-wide scan for linkage.

Significant LOD scores were detected for microsatellite markers on chromosome 9 (maximum LOD = 4.07) and recombination events delimited the *H* locus to a 3.2 Mb region (Clark *et al*. 2008)(Clark *et al*. 2008). The region harbors 20 genes, none of which is known to have a role in pigmentation.

In the dog, interbreed linkage disequilibrium (LD) is short and is therefore the preferred method for high-resolution mapping of identical-by-decent mutations that are common to several breeds (Karlsson *et al*. 2007). Nevertheless, the selection of genetically diverse individuals can allow for the use of LD in fine-mapping studies of phenotypes that are found predominantly in a single breed (Bannasch *et al*. 2008; Kukekova *et al*. 2009). In the present study, meiotic recombination events and LD mapping using unrelated harlequin dogs define the *H* locus in the Great Dane breed. Harlequin Great Danes are obligate heterozygotes; therefore, a critical interval was determined by identifying the region in which only one haplotype is in LD with the phenotype. Sequence analysis of the only gene in the region, *PSMB7*, reveals a single coding mutation that is heterozygous in all phenotypic harlequin Great Danes. We present here fine mapping of the *H* locus and analysis of a candidate causal mutation in *PSMB7*.

2. Materials and Methods

2.1. Sample collection

DNA samples isolated from whole blood or buccal cells from Great Danes and other pure bred dogs were available from previous and unrelated studies. Private owners of Great Danes submitted buccal cells from kindreds segregating harlequin and merle, and from unrelated dogs (no common grandparents) of various coat patterns and colors in

the general population. Genomic DNA was isolated using the Gentra Puregene Blood Kit (Qiagen, Valencia, CA, USA). Three nuclear families collected were comprised of 16 harlequins, 13 merles, and 4 solid Great Danes.

Skin tissue was submitted from five harlequin and three merle Great Dane puppies. Samples were acquired from ear tissues removed during routine cropping procedures and stored in RNA later® (Ambion, Austin, TX, USA) at -20°C. Total RNA was isolated using the RNA STAT-60^{TM} (Tel-Test, Inc., Friendswood, TX, USA) protocol. RNA was treated with DNA-free DNase (Ambion). All samples were collected following protocols approved by the Clemson University Institutional Review Board (#IBC2008-17).

2.2. Marker identification and genotyping

Microsatellites located within the candidate region on chromosome 9 were identified using the dog genome assembly (CanFam 2.0). Forward primers were fluorescently labeled with 6FAM (Suppl. Table 1). PCR amplification of all microsatellite markers was carried out as previously described (Clark *et al*. 2004). Products were resolved on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) with the GeneScan 500 LIZ internal size standard (Applied Biosystems). Genotypes were determined using Genemapper® Software v3.5 (Applied Biosystems).

Eighteen SNPs were selected from CanFam 2.0 and seven SNPs and five indels were identified during sequencing. Primer sequences are listed in Supplementary Table 2. PCR amplicons were analyzed by electrophoresis on agarose gels. Products were purified via gel extraction (QIAEX II Gel Extraction Kit, Qiagen) or by adding 0.5 units

Exonuclease I (New England Biolabs, Itswich, MA, USA) and 0.25 units of shrimp alkaline phosphatase (Promega, Madison, WI, USA) and incubating for 30 min at 37°C followed by 20 min at 80°C. Nucleotide sequencing was accomplished using the Big Dye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems). Products were purified using Spin-50 mini-columns with water (BioMax, Inc., Arnold, MD, USA) and resolved on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems).

2.3. Analysis of *PSMB7*

All primer sequences used in the amplification of *PSMB7* are shown in Supplementary Table 3. Amplification of the promoter region, all exons, and splice sites of *PSMB7* was carried out from genomic DNA. Primers were also designed to capture complete cDNA sequence. cDNA synthesis from RNAs was accomplished using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For RACE, cDNA was synthesized using the SMART^{TM} RACE cDNA Amplification Kit (Clontech, Mountainview, CA, USA), and product amplification was achieved using the Advantage 2 PCR Kit (Clontech). All sequences were aligned using CLUSTAL W (www.ebi.ac.uk/clustalw).

Primers for quantitative RT-PCR (qRT-PCR) were tested to ensure amplification of a single PCR product. Reactions were prepared according to the iQ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) protocol and performed using a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories). All reactions were carried out in triplicate, and quantification of gene expression was achieved by normalization against *GAPDH*. The comparative C_t method, also known as

the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta C_t = \Delta C_{t,sample} - \Delta C_{t,\ reference}$, was used to report the differential gene expression.

3. Results

3.1. Fine mapping of the *H* locus

Genotype data for four microsatellite markers (GAAA1, FH2885, GAAA2, and Ren287G01) that define or are within the *H* locus (Clark *et al*. 2008) were generated for newly acquired families of harlequin and merle Great Danes. In one family, a recombination event between the *H* locus and FH2885 was detected, narrowing the *H* locus to a 2.6 Mb region. To further refine this region, four new microsatellites between FH2885 and Ren287G01 were identified and genotyped for families with informative recombination events. Three markers centromeric to the *H* locus were genotyped in a family comprised of six harlequin and two merle dogs. One telomeric marker was genotyped in three families, collectively comprised of 13 harlequin, 9 merle, and 4 solid Great Danes. Two recombinant chromosomes (Suppl. Figures 1 and 2) delimited *H* to a 1.23 Mb region on chromosome 9, defined by the CT/CA and CA microsatellites (Fig. B-2A). This region contains 11 genes.

Nine unrelated Great Danes (8 harlequins and 1 merle) were genotyped for 30 SNPs and indels across the 1.23 Mb *H* interval. A 226 kb harlequin haplotype was identified and defined by SNPs 61.827 and 62.053. This region contains three genes: *GPR144*, *PSMB7*, and *NEK6* (Fig. B-2B). We could not further narrow the critical

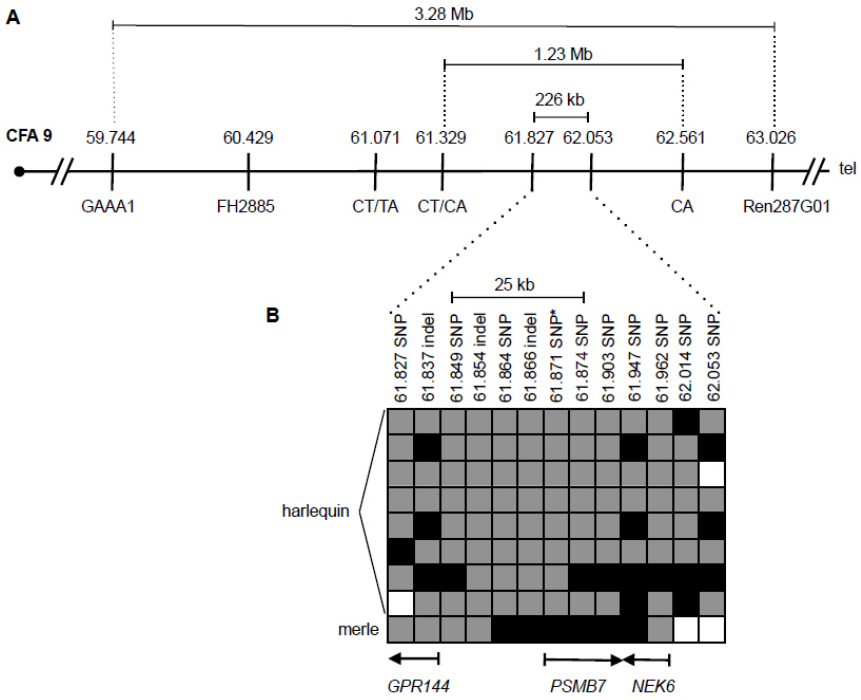

Figure B-2. Genetic mapping of the *H* locus in the Great Dane. (A) Physical map of chromosome 9 showing the location of microsatellite and SNP markers. Microsatellite name or repeat sequence is shown below the line and directly above is the position in Mb. *H* was previously mapped to a 3.28 Mb region. Recombination mapping using microsatellites refined the locus to 1.23 Mb. Association mapping using SNPs and indels revealed a 226 kb harlequin haplotype. (B) Genotypes for markers defining and within the 226 kb harlequin haplotype block are represented for the 8 harlequin and 1 merle dogs used to fine map *H*. Homozygosity is denoted by black for the major allele and white for the minor allele. Grey boxes indicate heterozygosity. The names of the three genes in the haplotype region and their locations relative to the markers are shown below the genotypes. The 25 kb block of heterozygosity in harlequin dogs is marked. The coding SNP in *PSMB7* is indicated by an asterisk.

interval using conventional homozygosity mapping because *HH* is early embryonic lethal and all harlequin dogs are obligate heterozygotes. Alternatively, we identified a single 25 kb haplotype block wherein all harlequin dogs are heterozygous. A homozygous interval in the merle dog (*hh*) overlaps this region. This homozygous merle haplotype was used to determine the wild-type chromosome of harlequins. The alleles of the wild-type haplotype are in agreement with the canine reference sequence, generated from a boxer (Lindblad-Toh *et al*. 2005). Therefore, we infer that the non-wild-type haplotype identified in harlequin dogs is found on the harlequin chromosome. The 25 kb region of heterozygosity contains the promoter region and first 4 exons of *PSMB7*.

3.2. Sequence and expression analysis of *PSMB7*

Sequence data from four Great Danes (three harlequin and one merle) were generated for the promoter region, all eight exons, splice sites, and flanking intronic regions of *PSMB7*. Only two heterozygous polymorphisms were present in all three harlequin dogs and absent from the merle dog: a T to G transversion in exon 2, and one intronic SNP. The intronic SNP, located 50 bp downstream from the coding SNP, was detected in two merle Australian shepherds and thus eliminated as a candidate mutation.

Additional sequence data covering 92% of the 25 kb heterozygous block, including 21.5 kb 5' of *PSMB7* and introns 1 through 3, were generated for a harlequin dog (Suppl. Figure 3). Thirty-six of 113 polymorphisms identified were heterozygous (Suppl. Table 4), and six of these were previously described in other breeds. The 30 remaining variants fell into three groups: 19 SNPs, 6 indels, and 5 repeat polymorphisms. Twenty-nine variants were considered unlikely to affect transcriptional regulation or

expression of *PSMB7* based on Dog/Human/Mouse/Rat Multiz conservation scores obtained from the UCSC dog genome browser (Fujita *et al*. 2011). An insertion (16 bp segmental duplication) genotyped in our haplotype analysis occurred in a region showing cross-species conservation but was heterozygous in a merle dog and thus eliminated as a candidate.

Quantitative RT-PCR was used to evaluate *PSMB7* mRNA levels in skin from five harlequin (*MmHh*) and three merle (*Mmhh*) Great Danes. No significant difference in *PSMB7* expression was detected (-1.17 fold change in harlequin dogs). Sequencing of the complete cDNA for *PSMB7* from a harlequin dog confirmed that both wild-type and mutant alleles are transcribed and that no other mutations are present (GenBank accession nos. **GU305912** and **GU305913**).

3.3. Analysis of the c.146T>G mutation

The variant, c.146T>G, was analyzed in 247 Great Danes and 104 dogs from other breeds. All harlequin Great Danes (n = 102) were heterozygous for the SNP and all merle dogs (n = 48) were homozygous wild-type. Fifty-nine percent of black (*mm*) Great Danes (n = 71) were heterozygous for the variant. Non-merle dogs (n = 81) representing 43 different pure breeds do not have the allele. None of the 351 dogs genotyped was homozygous for the c.146T>G mutation (Table B-1). Two merle Great Danes with white markings did not have the variant or share the harlequin haplotype.

The c.146T>G mutation occurs in the second exon, causing a valine to be replaced with a glycine at the 49th residue. To assess the significance of this mutation, we utilized PANTHER, a web service that predicts the functional consequences of amino

acid substitutions. PANTHER assigned the p.Val49Gly variant a score of -6.86 (results < -3.0 are considered functionally significant with more negative results indicating a higher likelihood that the substitution will impair protein function) and a 0.98 probability of being deleterious (Thomas *et al*. 2003; Thomas *et al*. 2006). In contrast, a p.Val49Ile variant observed in other species was predicted to be neutral, with a score of -2.80 (Smyth and Belote. 1999).

Table B-1 Distribution of genotypes at nucleotide 146 of PSMB7 among 247 Great Danes and 104 dogs from other breeds

		% Genotype		
Phenotype	n	T/T	T/G	G/G
Great Dane cases (*M-Hh*)				
Harlequin (*Mm*)	88	0	100	0
White (*MM*)	14	0	100	0
Merle controls (*M-hh*)				
Great Danes	25	100	0	0
10 other breeds	23	100	0	0
Non-merles (*mm-h*)				
Great Danes				
Black	71	41	59	0
Fawn	30	97	3	0
Brindle	11	91	9	0
Blue	8	88	12	0
43 other breeds	81	100	0	0

4. Discussion

Harlequin, a bigenic coat pattern exclusively selected for in the Great Dane breed, is caused by mutations in *SILV* and *H*. *SILV* is known to have an integral role in the development of melanocytes and harbors mutations that cause pigmentary defects in several species (Brunberg *et al*. 2006; Kerje *et al*. 2004; Kuhn and Weikard. 2007; Kwon *et al*. 1995; Schonthaler *et al*. 2005). Linkage mapping for the Great Dane unexpectedly placed *H*

within a segment of chromosome 9 that lacked genes known to influence pigmentation. Haplotype analysis and heterozygosity mapping indicated that *H* is located in a 25 kb region containing partial coding sequence for only one gene: *PSMB7*, a component of the proteasome. All phenotypic harlequin Great Danes harbor a heterozygous SNP (c.146T>G) in exon 2 of *PSMB7*. The variant is absent from all merle dogs and, although present in nearly 60% of our Great Dane population, is never observed in a homozygous state. The mutation was not detected in any other breeds tested herein, including ten breeds having merle coat patterning. Based on these data, we propose that an alteration of the proteasome is the functional basis for the harlequin pattern of the Great Dane. Direct mutation of the proteasome has not previously been associated with pigmentary traits; however, the abnormal processing and/or trafficking of mutant proteins by the ubiquitin proteasome system is hypothesized to play a role in hypopigmentary diseases such as oculocutaneous albinism (Ando *et al*. 2009).

Protein degradation is an essential part of many biological processes, including cell cycle progression, DNA repair, apoptosis, and stress response. The ubiquitin proteasome system represents the primary pathway for regulated protein degradation (Tanaka. 2009). Proteins scheduled for turnover and those damaged by gene mutations or misfolding undergo a post-translational modification in which ubiquitin monomers are covalently attached to lysine residues in the target protein, thereby labeling them for degradation (Ravid and Hochstrasser. 2008). The 26S proteasome is the large complex that recognizes poly-ubiquitinated proteins and breaks them down into short peptides. At the core of the complex is the 20S proteasome, comprised of seven α and seven β subunits

arranged into four stacked rings. Three subunits (β1, β2, and β5) of the two inner β-rings have catalytically active threonine residues at their N termini that perform the proteolysis. The β2 subunit provides trypsin-like activity to cleave peptide bonds at the C-terminal side of basic amino acid residues and is encoded by *PSMB7* (Tanaka. 2009).

The β2 subunit is synthesized as an inactive precursor with an N-terminal propeptide. The predicted full-length canine PSMB7 protein includes a 43 amino acid propeptide that is removed in an autocatalytic reaction to yield a mature peptide of 235 amino acids. The mature peptide sequence of human PSMB7 is well-conserved with the yeast homologue, PUP1 (Tanaka. 2009); human and canine sequences show 97% identity. The c.146T>G mutation causes a glycine to be substituted for a valine, thus replacing a large hydrophobic side chain with hydrogen. The valine is the sixth residue of the mature peptide and is highly conserved (Fig. B-3), although substitution with isoleucine, which also has a large hydrophobic side chain, has been observed in several non-mammalian organisms, including *Drosophila melanogaster* (Smyth and Belote. 1999).

β2 is the first subunit incorporated during β-ring assembly (Tanaka. 2009). Both the N-terminal propeptide and C-terminal extension of the β2 subunit are necessary for proper proteasome assembly; in human cells, deletion of either domain is lethal (Ando *et al*. 2009; Arendt and Hochstrasser. 1999; Jager *et al*. 1999; Ravid and Hochstrasser. 2008). In *D. melanogaster*, a missense mutation at residue 170 of *PSMB7* causes early larval lethality in homozygotes (Smyth and Belote. 1999). Heterozygotes exhibit a temperature-sensitive phenotype, with no visible phenotypic effect when kept at 25°C but significant developmental defects at 29°C (Smyth and Belote. 1999). The authors propose that the

PSMB7 mutation has a dominant negative effect and disrupts the function of the entire proteasome (Smyth and Belote. 1999)). Additionally, in vertebrate cells, it was shown that disruption of the β2 subunit causes an accumulation of poly-ubiquitinated proteins *in vivo*. This provides further evidence supporting a critical role for maintaining integrity of the proteasome (Tanahashi-Hori *et al*. 2003) .

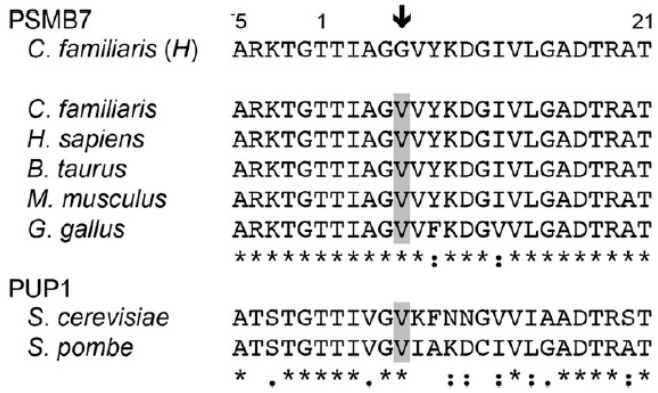

Figure B-3. Protein sequence alignment of PSMB7/PUP1. The missense mutation in harlequin dogs occurs at the sixth position of the mature peptide, indicated by the arrow. Amino acid conservation between several species is shown below the PSMB7 sequence. Conservation between PSMB7 sequences and the yeast homologue, PUP1, is shown below the PUP1 sequence. Asterisks indicate identical residues while colons and periods denote conservative and semi-conservative substitutions, respectively.

To our knowledge, harlequin is the first naturally occurring phenotype attributed to mutation of the β2 subunit. Because *HH* is not viable and because studies have shown that loss of trypsin-activity per se is not lethal (Arendt and Hochstrasser. 1997; Heinemeyer

et al. 1997), we hypothesize that the p.Val49Gly variant causes a dominant-negative effect by compromising the assembly and/or function of the proteasome complex. Each 20S proteasome contains two β2 subunits; 75% of proteasomes in an *Hh* dog will presumably have one or two mutant components. The absence of a detectable phenotype in *mmHh* dogs suggests that the harlequin pattern of *MmHh* dogs results from an interaction between mutant SILV and the impaired proteasome. Direct interaction of SILV and the proteasome has been previously reported (Vigneron *et al*. 2004).

The *M* allele of *SILV* yields mutant transcripts with large insertions (Clark *et al*. 2008), presumably resulting in aberrant proteins targeted for degradation. The dilute background of merle dogs (*Mmhh*) is the result of haploinsufficiency: a single wild-type allele is insufficient for normal pigmentation. Additional studies are necessary to determine how, in the presence of mutant SILV, the *H* allele of *PSMB7* interferes with the function of wild-type SILV to cause the amelanic background of harlequin dogs (*MmHh*). One possible explanation is that proteasome function is impaired and unable to meet the increased demand for proteasome activity created by accumulating defective SILV. Mutant SILV may then reach toxic levels and cause death of melanocytes, or could exert a dominant-negative effect by creating a multimer with wild-type SILV, preventing the formation of a functional fibril matrix.

Acknowledgements

We are grateful to the many dog owners who submitted samples for this work. We also wish to thank JP Yousha, Kathleen Davis, Judith Arsenault, and Leslie and Jay Eaton for providing photos used in Figure 1.

Supplementary information for this manuscript is available online at http://www.sciencedirect.com/science/article/pii/S0888754311000164

Appendix C

Exclusion of COL2α1 in canine Legg-Calvé-Perthes Disease*

Source/description: Legg-Calvé-Perthes Disease (LCPD) is a developmental disease characterized by osteonecrosis of the coxofemoral joint in young dogs (<18 months) (Demko and McLaughlin. 2005). LCPD most commonly occurs in breeds of small stature, *i.e*., toy, miniature, and terrier breeds. Radiographic evidence of lucency in the femoral head and articular incongruency are hallmarks used for diagnosis of LCPD; the etiology is currently unknown. LCPD most commonly occurs in breeds of small stature, i.e., toy, miniature, and terrier breeds. The West Highland White Terrier (WHWT) is one of 11 breeds with increased risk for development of LCPD (LaFond *et al*. 2002).

Canine LCPD is clinically and pathologically similar to LCPD in humans (Roperto *et al*. 1992). Two independent studies of LCPD in human families reported a mutation in *COL2A1* which segregates with the disease (Miyamoto *et al*. 2007; Su *et al*. 2008). Therefore, *COL2A1* was investigated as a candidate gene for canine LCPD.

Study design: Genomic DNA was extracted from whole blood of 38 WHWTs. Of these, 15 were diagnosed with LCPD by veterinarians. Sequencing primers were designed (Table S1), and PCR was performed following the manufacturer recommendations for ThermoPrime Master Mix with ReddyMix (ABgene). DNA sequences for the 54 coding exons and intron-exon junctions were determined by direct sequencing. Sequences were

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assembled for two WHWT: one LCPD-affected and one control. Polymorphisms among the two dogs and the CanFam2 reference sequence were examined in four to ten additional WHWTs in order to evaluate potential associations. Thirty-eight SNPs from the Affymetrix version 2 custom canine SNP array surrounding the *COL2A1* locus on chromosome 27 were selected for analysis. DNA preparation and genotype calls were completed following the manufacturer's Mapping 500K Assay protocol (http://www.affymetrix.com/support/downloads/manuals/500k_assay_manual.pdf). The whole-genome association analysis program PLINK (Purcell *et al*. 2007) was used to analyze genotypes for association with LCPD

Analysis: Sequencing of *COL2A1* revealed 18 SNPs and indels, but none segregated with the affected dogs. Additionally, SNPs across a 1.9 Mb region including *COL2A1* were not in linkage disequilibrium with LCPD (Table S2). We expected to see a co-segregation of SNPs with disease if *COL2A1*exerted a major effect. A regulatory mutation is unlikely. This study suggests that a mutation of *COL2A1* is not responsible for LCPD in WHWT.

Acknowledgements: Owners of WHWT are thanked for providing samples. This work was supported by the Westie Foundation of America, Inc. and the AKC Canine Health Foundation. The authors additionally wish to thank the Clemson University Genomics Institute for use of the Affymetrix GeneChip platform.

Supplementary information for this manuscript is available online at http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2052.2011.02215.x/suppinfo

Appendix D

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