# CHARACTERIZATION OF THE MUTATION CAUSATIVE FOR AUTOSOMAL RECESSIVE HEREDITARY NEPHROPATHY IN THE ENGLISH COCKER SPANIEL AND ANALYSIS OF GENE EXPRESSION IN MULTIPLE MODELS OF HEREDITARY NEPHROPATHY

A Dissertation

by

ASHLEY GREENE DAVIDSON

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

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Genetics

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Approved by:

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Major Subject: Genetics

# ABSTRACT

Characterization of the Mutation Causative for Autosomal Recessive Hereditary Nephropathy in the English Cocker Spaniel and Analysis of Gene Expression in Multiple Models of Hereditary Nephropathy. (May 2007)

Ashley Greene Davidson, B.S., New Mexico State University

Chair of Advisory Committee: Dr. Keith E. Murphy

The domestic dog, *Canis familiaris*, has over 450 naturally occurring inherited diseases. Over half of these diseases are clinically similar to human diseases making the dog an excellent model in which to study human hereditary diseases. Alport syndrome (AS), a group of heterogeneous, hereditary renal diseases, is one example of such a human disease. The disease is transmitted in three fashions: X-linked, autosomal recessive, and autosomal dominant.

AS is caused by mutations in *COL4α3*, *COL4α4* or *COL4α5*, all members of the type IV collagen family. The proteins products of these genes along with those of the other type IV collagen family members (*COL4α1*, *COL4α2*, and *COL4α6*) are structural components of basement membranes throughout the body. This dissertation describes the measurement of mRNA transcripts in two canine models of AS: a mixed breed model of X-linked AS (XLAS) and the English Cocker Spaniel (ECS) model of autosomal recessive AS (ARAS). The work done revealed a decrease in *COL4α4* transcripts.

The similarity between the decrease of *COL4α5* in the XLAS model and that for *COL4α4* in the ARAS model lead to the investigation of *COL4α4* as the gene harboring

the mutation causative for ARAS in the ECS. Upon sequencing *COL4α4*, the causative mutation was determined to be an A to T transversion in exon 3.

To provide an *in vitro* model to study type IV collagens, a protocol was designed and experimentally validated to isolate and culture canine Sertoli cells. Canine testes cells were isolated and cultured. Cells were verified as Sertoli cells through positive identification of both SOX9 and Clusterin B proteins, along with sequence verification of SOX9 transcripts. This *in vitro* model provides a tool to further study the type IV collagens.

Overall, the research described herein lead to the identification of the mutation causative for ARAS in the ECS. With this knowledge a genetic test was developed to test for the disease. This research also provided valuable information about the transcript levels of type IV collagens in two models of AS, and provided a novel model in which to study the type IV collagens further.

# DEDICATION

For my parents

Thank you for everything

## ACKNOWLEDGMENTS

I would first like to thank my advisor, Dr. Keith Murphy, for giving me the opportunity to work on this project and to learn the ups and downs of research while earning my degree. I would also like to extend thanks to my committee members, Dr. George Lees, Dr. Ann Kier, and Dr. Charles Long for their help along the way.

All the members of the Canine Genetics Laboratory at Texas A&M University deserve special thanks as well. Specifically, I want acknowledge Rebecca Bell for all her help in both my research and writing. Many members of the laboratory deserve a personal thanks, including, Stephanie Herbst, Jacquelyn Wahl, Jessica Moody and Michelle Boggs. Without their support and friendship I would not be where I am today.

All the work described within this dissertation was made possible by support from the English Cocker Spaniel Club of America. My gratitude also goes out to the English Cocker Spaniel owners and breeders who have donated samples from their dogs throughout the past 14 years. Among these individuals, both Addi Pittman and Sandy Platt deserve to be further recognized. The time, effort, and money they have contributed to this work is invaluable.

The support I have received from my family is immeasurable. My parents, George and Maureen Davidson, have been behind me, encouraging and pushing me, throughout my entire education. Not only has my father supported my education, he has played a valuable role in it. I want to thank him for all the help he provided scientifically. My husband, John Hamlett, has stood by me through some of the more testing times in the past few years, I am lucky to have him and I thank him.

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# CHAPTER I

# INTRODUCTION

## The dog as a model system

The domestic dog, *Canis familiaris*, is an especially useful animal model for study of human hereditary diseases. While diseases mimicking those found in humans can be induced for study in conventional model organisms (*e.g.*, mouse), the dog has over 450 naturally occurring hereditary diseases. [1]. More than half of these have the potential to be studied as models for a virtually identical human disease. These include both monogenic and multifactoral diseases [1, 2], and range from rare diseases such as narcolepsy and Alport syndrome to more common diseases such as arthritis and cataracts. The genes and specific mutations for many of these monogenic diseases have been elucidated. Importantly, using the dog as a model also provides a greater understanding of the disease and therefore, will help to improve the quality of life of the dog.

Several of the benefits of canine models branch from the specific role dogs play in our culture as a domesticated pet, as opposed to being a laboratory animal. In this role, the dog has the highest level of medical surveillance of any non-human species [2]. Not only do we give our dogs top-notch medical care, we also co-habitate with them, often sharing the same food and bed. A shared environment can act as a valuable control in studying diseases which may have environmental influences. Also, our interest in our dog's heritage and desire to keep accurate breeding records has led to the maintenance of extensive and accurate multigenerational pedigrees, a very desirable trait in a model

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This dissertation follows the style of *BMC Biology*.

organism because they facilitate the study of hereditary traits, including diseases.

Because of its evolution and the present-day breeding practices, many breeds exhibit unique intra-breed homogeneity but inter-breed diversity. This phenomenon and the desire to propagate specific and individual breeds, along with their particular characteristics, has lead to the development of breed clubs. Today the American Kennel Club (AKC) recognizes over 150 breeds [3]. However, variation can be seen among more than just the registered breeds and there are over 1000 different variations (or breeds) of the dog world wide [4]. These stem not from natural selection but rather artificial selection by humans, as we carefully handpick individuals for propagation based on the role, personality, or physical characteristics desired for the particular breed. This breeding strategy, along with the breed club's registration requirements (that both parents of a registered dog must be registered within the breed as well), has created a barrier to gene flow, leaving each breed as a genetically isolated population which can be a great advantage in genetic studies.

Also adding to intra-breed genetic homogeneity is the low number of founders for some breeds, along with bottleneck and popular sire effects. Bottlenecking occurs when a small population of individuals is left to continue the breed; this has been seen after large disasters or catastrophic events that wipe out a large number of the breeding population. The popular sire effect, on the other hand, is the result of the popularity a particular stud attains after exhibiting exceptional breed standards. Breeders wish to introduce such sires' desirable qualities in their lines, and if a single sire is brought into enough lines, this increases the already high level of genetic homogeneity.

**Tools available to study the dog**

The inherent benefits, as described above, to studying the dog as model have been known for decades; however, only within the past few years have there been the necessary scientific advances to allow researchers to study the dog at the genetic level. Although research involving the domestic dog was slow at first, *i.e.*, the complete karyotype of the dog was not available until 1999, research since then has rapidly advanced [5]. One of the major advances in canine genomics came in 2003, when the reference sequence consisting of 1.5X sequence coverage of the canine genome was published [6]. That same year a White Paper was submitted to, and funded by, the National Human Genome Research Institute (NHGRI) proposing to go beyond the reference sequence already published and undertake the sequencing of the entire dog genome. The project's completion came with the sequence publication in 2005. The result was a 7.5X sequence coverage comprised of 31.5 million shotgun sequence reads [7]. The sequence covers ~99% of the euchromatic regions of the genome and over 99% has been correctly assembled. The sequence was assembled to span 2.41 Gb with only 1% remaining as gaps. From this assembly, sequence was arranged and oriented on the 38 canine autosomes and the X chromosome, however, about 3% could not be assigned a chromosomal position due to their highly repetitive nature [7].

The full sequence and details of its assembly are publicly available on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). There, a user can search the deposited sequence using the site's BLAST program, identify predicted genes or coding sequences from the assembly, compare genomic and coding sequences, and align unknown or predicted

sequences to the known genome sequence. These tools allow a researcher to obtain important information about the genes and putative proteins, they are studying.

These requisite tools for canine research have added to the advantages of studying the dog. They have also allowed investigators to make some fundamentally important discoveries and observations about mammalian genomes. For example, the completion of the 1.5X sequence revealed that the genetic distance between the human and dog is half that of the human and mouse, as assessed by genomic alignments [6]. And by comparing the canine sequence to that of other mammals, it is possible to perform more informative comparative analyses. For example, further understanding of the evolutionary shaping of mammalian genomes through chromosomal rearrangements, genomic insertion and deletions, and nucleotide divergences has been gained [7].

**Type IV collagens**

The collagen superfamily is comprised of over 20 gene families, the most recently discovered being collagen type XXVIII [8]. All collagens can be divided into two distinct groups: fibril forming and non-fibril forming. These structural proteins are found throughout the body, from bone and cartilage to blood vessels and muscles. Specifically, the type IV collagens, categorized as non-fibril forming, are involved in the formation of extracellular (or basement) membranes of tissues including, but not limited to, the kidney, ear, eye, and testes [9-13]. This collagen family plays an important role not only in cell and tissue structure through their integration into basement membranes, but also in all the functions associated with those membranes [14].

The type IV collagen family is comprised of six distinct alpha chains (α1-6). The genes encoding these proteins are uniquely located in a head-to-head fashion on opposite strands of three canine chromosomes. Specifically, *COL4α1*and *COL4α2* are located on canine chromosome 22 (CFA22), *COL4α3*and *COL4α4* on CFA 25, and *COL4α5* and *COL4α6* on the CFA X [15]. These correspond to human chromosomes 13, 2, and X, respectively [16-20]. This unique genomic structure stems from the duplication and inversion events during the evolution of the type IV collagen gene family [21]. Because of these duplication events, the type IV collagen genes have a marked sequence identity. However, they can be split into two separate groups: α1-like, and α2-like. The α1-like genes, as the name suggests, were all duplicated from the *COL4α1* gene and are comprised of *COL4α1*, *COL4α3*, and *COL4α5* genes, and the α2-like genes, duplicated from the *COL4α2* gene, contain the *COL4α2*, *COL4α4*, and *COL4α6* genes [17, 21, 22].

Collagen proteins encoded by these genes are composed of three distinct domains: a hypervariable NC1 domain at the carboxy terminal, a long collagenous region in the middle of the protein, and a short 7S domain at the amino terminal [13, 23-26]. Each alpha chain made is incorporated into trimers which link together to form the framework for basement membranes. To do this, the recognition sequence within the NC1 domain dictates which three proteins partner to comprise the trimer [27, 28]. The trimers are then linked together in pairs by their NC1 domains to create hexamers. Four of these hexamers are linked at the 7S domain to form the network found in membranes (figure 1) [26, 29, 30]. The collagenous domain is made of multiple repeats of the amino acid sequence Gly-X-Y. These repeats are periodically interrupted to give the helices flexibility [31, 32]. Defects in any one of the three alpha chains in any heterotrimer prevent proper assembly

and subsequent deposition, of any of the proteins involved, into their respective basement membrane [28].

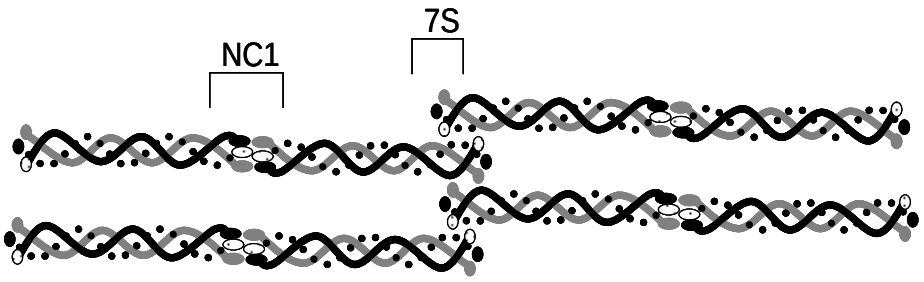


**Figure 1.** Pictorial representation of collagen network in basement membranes. The NC1 domain (indicated on figure) links two trimers together and the 7S domain (indicated on figure) links four of the hexamers together.

Although there are 720 possible trimer combinations for the six type IV collagen proteins, these trimers only follow a pattern of two α1-like and one α2-like proteins in one trimer. Further, there are only three distinct trimers actually produced and found in basement membranes: α1.α1.α2, α3.α4.α5, and α5.α5.α6 [33-35]. These heterotrimers are expressed in a spatially conditional pattern in basement membranes throughout the body. The α1.α1.α2 trimer is ubiquitously found in the body, while the α3.α4.α5 trimer is limited to membranes in the ear, eye, lung, testes and the glomerular basement membrane (GBM) of fetal and adult kidneys. The α5.α5.α6 heterotrimer is found the basement membranes of the skin, smooth muscle, esophagus, testes and Bowman's capsule in the kidney [11, 36, 37].

**Alport syndrome (AS) and hereditary nephropathy (HN)**

Alport syndrome (AS) describes a group of hereditary nephropathies affecting the GBM that occur in the human. Hereditary nephropathy (HN) is the most accurate term to

describe the corresponding group of diseases in the dog. While HN is currently the most exact term for the canine form, it has been referred to by many different terms, such as familial nephropathy (FN). Therefore, all three terms have been used interchangeably when discussing the disease.

Although the dog represents an excellent natural model for AS, there are a few distinctions between human AS and canine HN. The most significant of these differences is the fact that HN does not display the multi-organ symptoms that AS patients can present with, such as sensorineural deafness and ocular lesions [38-41]. Both AS and HN are, however, characterized by distinctive ultrastructural changes to the GBM and progressive renal failure. They are caused by mutations in the genes that encode members of the type IV collagen proteins found in these basement membranes, as described above. AS and HN specifically affect the GBM of the kidney, causing a distinctive multilaminar splitting of the GBM ultrastructure, a phenomenon typical of this disease. This contributes to the development of hematuria, proteinuria, and progressive renal injury, which eventually lead to end stage renal disease (ESRD) [30].

AS is transmitted in three fashions: X-linked (XLAS), autosomal recessive (ARAS), and autosomal dominant (ADAS). XLAS is caused by mutations in *COL4α5*, located on the X chromosome in both the human and the dog, and is the most common form of AS, accounting for approximately 85% of human cases [15, 19, 42]. More than 300 mutations causative for human XLAS have been described [43]. ARAS occurs less frequently, accounting for about 15% of human AS cases, and is caused by mutations in either *COL4α3* or *COL4α4* [42, 44]. These genes are located on human chromosome 2

and canine chromosome 25 [15, 45]. Mutations in *COL4α3* or *COL4α4* have also been reported to cause ADAS, the rarest form of AS [46-49].

The type IV collagen heterotrimer consisting of α3.α4.α5 is essential to the structure and function of the adult GBM, and, when absent, results in AS and HN [50]. As described above, the absence of any one of the three proteins in the trimer leads to the absence of the entire trimer in the basement membrane. Therefore, both XLAS and ARAS are characterized by the absence of COL4α3, COL4α4, and COL4α5 in the GBM of affected individuals.

**Animal models of AS**

While the underlying causes and modes of transmission of AS are well known, many aspects of the disease remain to be understood, and renal transplant is the only treatment option. Further understanding of this disease can be achieved through the use of available animal models (table 1). There are both murine and canine models for the two most common forms of AS (*i.e.*, XLAS and ARAS) [38-40, 51-55].

Two natural models of XLAS are found in the dog. The first, in a Samoyed kindred, was described in 1984, and in 1994 was determined to result from a G to T nucleotide substitution in the *COL4α5* gene [40, 56]. Upon immunofluorescence (IF), these dogs show an absence of the trimer composed of α5.α5.α6 and decreased levels of the α3.α4.α5 trimer in the GBM [57]. Another XLAS canine model was described in a mixed breed originating in Navasota, Texas (and thus termed NAV dogs) in 1999 [39]. The causative mutation, a 10 base pair deletion in exon 9 of *COL4α5*, was described in 2003 [58]. The type IV collagen staining for the these dogs is similar to that of the

Samoyed, specifically, in the GBM there is an absence of the α5.α5.α6 trimer and a decrease of the α3.α4.α5 trimer [39]. Recently, a third XLAS animal model, a murine model, was created by inducing the causative human mutation G5X in the murine *COL4α5* gene [52]. Immunohistochemical studies on these mice reveal that α5 expression is lost along with that of α3 in the renal basement membranes [52].

Murine models make up the majority of the ARAS models, with only a single naturally occurring canine model. HN has been described in the English Cocker Spaniel (ECS) for 50 years [59]. However, only very recent work reported in chapter III identified the causative mutation. A total of three murine models of ARAS have been generated. Two of these are targeted knockout models of the *COL4α3* gene, while the third involves a transgenic deletion encompassing part of both the *COL4α3* and *COL4α4* genes [53-55]. For all animal models of ARAS, the α3.α4.α5 trimer is completely absent from the GBM, as assessed by IF [38, 53-55]

Two canine models of ADAS remain the only animal models of this form of AS described to date. The first of these to be identified was in 1995 when Hood and colleagues described a hereditary nephropathy transmitted in an autosomal dominant fashion in bull terriers [60]. The second, also described by Hood in 2002, occurs in Dalmatians [61]. The underlying mutations causing either of these forms of HN have yet to be described. Unlike the other forms of AS described above, IF shows positive staining for both α3,α4.α5 and α5.α5.α6 trimers in the GBM of both the bull terriers and Dalmatian affected with ADAS [60, 61].

**Table 1.** List of available animal models of AS. Form of AS, species of model, genetic cause, and reference are listed.

| Form of AS Modeled | Animal | Causative Aberration | Reference |
|---|---|---|---|
| XLAS | Canine (2) | | |
| | Samoyed | G to T, exon 35 | [40] |
| | NAV dogs | 10 bp deletion, exon 9 | [58] |
| | Murine (1) | Induced human G5X mutation (G to T, exon 1) | [52] |
| ARAS | Canine (1) | | |
| | English Cocker Spaniel | Reported in chapter III | chapter III |
| | Murine (3) | Targeted insertion of *Neo* cassette, exon 5 | [53] |
| | | Targeted deletion of first three NC1 exons | [54] |
| | | Transgene insertional mutation resulting in deletion of COL4α4 exons 1-12 and COL4α3 exons 1 and 2 | [55] |
| ADAS | Canine (2) | | [60] |
| | Bull Terrier | Uncharacterized | [61] |
| | Dalmatian | Uncharacterized | |

# CHAPTER II

# ANALYSIS OF TYPE IV COLLAGEN GENE EXPRESSION IN AUTOSOMAL RECESSIVE AND X-LINKED FORMS OF ALPORT SYNDROME IN THE ENGLISH COCKER SPANIEL AND MIXED BREED DOG*

## Background

AS refers to a progressive hereditary glomerular nephropathy caused by mutations in three members of the type IV collagen gene family that encode proteins required for normal glomerular basement membrane (GBM) structure and function. The condition occurs in the human and various breeds of the domestic dog and is transmitted in X-linked (XLAS), autosomal recessive (ARAS), and autosomal dominant (ADAS) fashions. Although the genetic and phenotypic spectra of AS are diverse, the primary structural abnormalities common to all forms of the disease are defects in the GBM. These abnormalities include a distinctive multilaminar splitting of the GBM, which contributes to the development of hematuria, proteinuria, and progressive renal injury. Additionally, sensorineural deafness and ocular lesions occur in many human AS cases [42, 62].

In the human, the genetic bases for the diseases are known for XLAS, ARAS, and ADAS. Mutations in *COL4α5* are responsible for XLAS and mutations in *COL4α3* or *COL4α4* are responsible for the autosomal forms of AS. These three type IV collagen

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* Portions of this chapter are reprinted in part with permission from Davidson A, Bell R, Lees G, Kashtan C, Davidson G, Murphy K: **Genetic cause of autosomal recessive hereditary nephropathy in the English Cocker Spaniel.** *JVIM* 2007, **21**

genes, along with the other three members of this gene family, encode six distinct peptides (α chains) that are assembled into three heterotrimers; α1.α1.α2, α3.α4.α5 and α5.α5.α6. These heterotrimers are distributed in basement membranes throughout the body [33-35]. In the kidney, the α1.α1.α2 and α3.α4.α5 heterotrimers are found in fetal and adult GBM, respectively [54]. The α3.α4.α5 heterotrimer is essential to the structure and function of the adult GBM, and, when absent, results in AS [50]. Therefore, both XLAS and ARAS are characterized by the absence of COL4α3, COL4α4 and COL4α5 in the GBM of affected individuals.

More than 95% of human cases are either X-linked or autosomal recessive, although rare cases of ADAS have been reported. Mutations in *COL4α5* result in XLAS, the most common form of AS, accounting for about 85% of human cases [19, 42, 63]. In both the human and the dog, *COL4α5* is located on the X chromosome [15, 19]. More than 300 mutations in the *COL4α5* gene causing human XLAS have been reported. ARAS, which accounts for about 15% of human cases, results from mutations in *COL4α3* or *COL4α4*, which are on chromosome 2 in the human and chromosome 25 in the dog [15, 64].

XLAS has been identified in two families of the dog: 1) a Samoyed kindred, and 2) a kindred of a mixed breed dogs, the NAV dogs, so named due to the city of their origin [39, 65]. In each family, XLAS arises from a unique mutation in *COL4α5*. In the Samoyed model of XLAS, the causative mutation is a single base substitution in exon 35, which results in a stop codon [40]. The causative mutation in NAV dogs is a 10bp deletion in exon 9 [58]. The deletion causes a frameshift leading to a premature stop codon in exon 10 resulting in a severely truncated protein. Affected dogs manifest

clinical abnormalities typical of canine AS, including onset of proteinuria before six months of age and rapid progression to uremia in males at six to eighteen months of age [39].

ARAS is present in the ECS. These dogs exhibit typical manifestations of AS, including distinctive thickening and splitting of the GBM, onset of proteinuria at five to eight months of age, and juvenile-onset renal failure [38]. As is the case for XLAS, this disease is characterized by abnormal GBM expression of type IV collagens, as demonstrated by immunofluorescence (IF) staining. The mutation causative for ARAS in the ECS is described in chapter III, but has been suspected to be in either *COL4α3* or *COL4α4,* as in human AS [46, 47, 66].

Characterization of the gene expression changes that occur in these two canine models of AS will enhance our understanding of the disease processes. Although gene expression profiles in other animal models of AS have been reported, no previous studies have utilized real time quantitative RT-PCR (qRT-PCR). Through the use of qRT-PCR, we identified the changes in expression of the type IV collagen genes of XLAS-affected, XLAS-carrier, and ARAS-affected dogs, compared with dogs having no signs of renal disease. These data suggest that *COL4α4* is the gene causative for ARAS in the ECS.

**Results and discussion**

This study utilized qRT-PCR to analyze type IV collagen gene expression in AS. Other work included the use of IF staining to assess protein expression as well as northern blotting and RNase protection assays to assess mRNA expression [38, 39, 52, 67]. To complement these data, qRT-PCR, a more sensitive method for assessment of

transcription, was performed. Data generated using this approach provide an enhanced insight regarding gene expression changes in the renal cortex of AS-affected subjects, and offer a useful comparison of gene expression changes between two different genetic forms of canine AS.

*XLAS-affected NAV dogs*

To assess gene expression changes in XLAS-affected NAV dogs at ESRD, comparisons were made to dogs, including normal NAV dogs, with no signs of renal disease. As shown in Figures 2 and 3, there was a greater than two fold down regulation of *COL4α5* (p=0.03) in XLAS-affected NAV dogs. No changes in gene expression levels were detected for *COL4α3*, *COL4α4*, and *COL4α6* (p=0.65, 0.88, and 0.54, respectively).

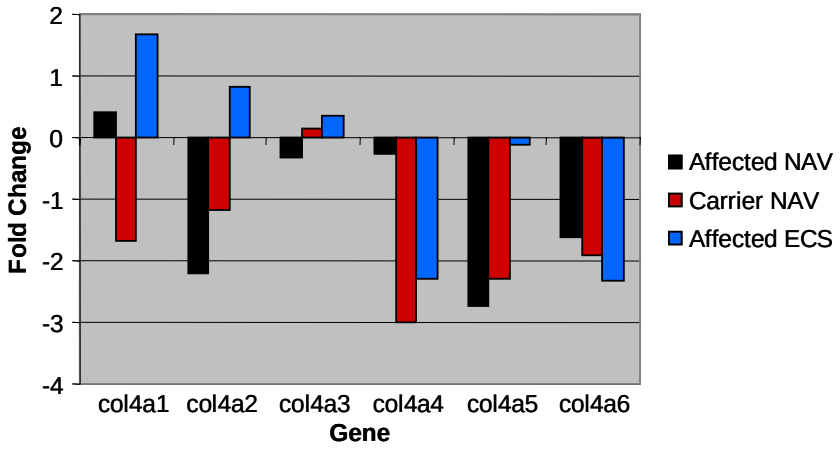


**Figure 2.** Results of quantitative real time RT-PCR analysis displayed by groups of dogs. Bars represent transcript levels of selected type IV collagen genes in the renal cortex of respective groups of dogs. Each bar shows the fold change in the designated group of affected dogs compared with the group of dogs without HN for the indicated gene.

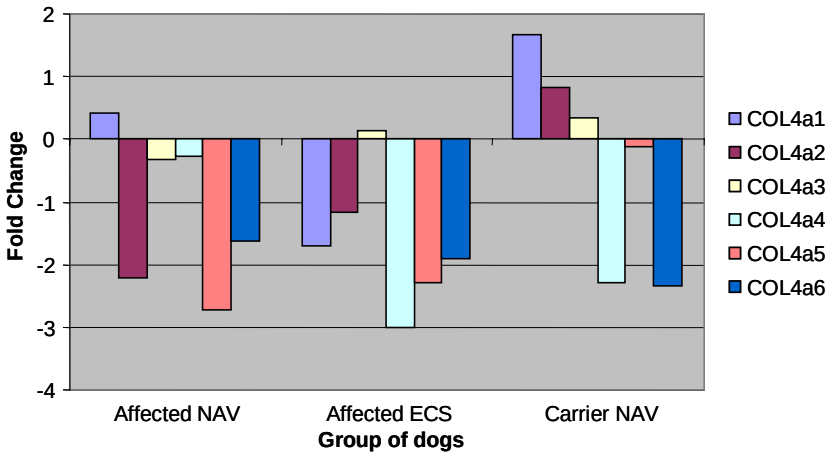


**Figure 3.** Results of quantitative real time RT-PCR analysis displayed by gene. Bars represent transcript levels of selected type IV collagen genes in the renal cortex of respective groups of dogs. Each bar shows the fold change in the designated group of affected dogs compared with the group of dogs without HN for the indicated gene.

Because the concentration of full length transcripts of *COL4α5* were reduced in XLAS-affected dogs, and because *COL4α3*, *COL4α4*, and *COL4α6* mRNA levels in XLAS-affected dogs were similar to levels in normal dogs, these data suggest that the absence of these proteins in renal basement membranes (previously demonstrated by IF [39]) is likely due to a post-translational event. That is, although COL4α3, COL4α4, and COL4α6 are properly translated, the absence of functional COL4α5 proteins prevents the formation of the α3.α4.α5 and α5.α5.α6 trimers and their subsequent deposition in renal basement membranes.

Expression patterns for the other type IV collagen genes differed from each other (Figures 2 and 3). That is, *COL4α1* exhibited no change in expression (p=0.69), whereas, *COL4α2*, exhibited a greater than a two fold down regulation (p=0.13). Although the p-value obtained for *COL4α2* is not strongly significant, it does support the trend of down

regulation. This is perplexing since staining for COL4α1 and COL4α2 in the GBM, Bowman's capsule (BC), tubule basement membrane (TBM), and interstitium of XLAS-affected NAV dogs is positive [39]. However, one possible explanation could be that although there is a down regulation of transcription, this is not coupled with an increase in the degradation of the proteins. Because there is an absence of the α3.α4.α5 trimer in all basement membranes, the COL4α1 and COL4α2 proteins are able to be deposited in high enough amounts to be detected by IF staining. Therefore, the positive staining results may be due to increased retention of these collagens in the basement membranes, not an increase in their transcription or translation. Also contributing to this could be the fact that the renal samples are from whole renal cortex not isolated GBM. Therefore, any transcript level changes in membranes or interstitium outside the GBM will be included in these measurements along with those within the GBM.

Although IF findings for XLAS-affected Samoyeds are similar to those for XLAS-affected NAV dogs [39, 50], the gene expression profiles for the Samoyed model notably differ from those of the NAV dogs. That is, the most recently published data for the Samoyed [67] report both *COL4α1* and *COL4α2* to be up regulated, *COL4α3* and *COL4α4* to be down regulated, *COL4α5* not detectable and *COL4α6* to have no change. With the exception of *COL4α5* and *COL4α6*, these data differ from the patterns reported here for the NAV dogs. The most striking difference is shown for *COL4α3* and *COL4α4*, for which no changes were detected in the NAV dogs (Figures 2 and 3). However, gene expression changes reported herein do agree with those published for the murine model of XLAS [52]. It is important to note that the mutations leading to XLAS are different for each of these three models. It has been reported that the more severe the mutation in

*COL4α5*, the more severe the disease outcome [68], which may explain the differences in gene expression for these two canine models of XLAS.

It is important to address the fact that the type IV collagen gene expression profiles for the XLAS-affected NAV dogs more closely mimic a murine model of XLAS than another canine model of the same disease. This may be due to the differences in causative mutations. That is, the XLAS-affected NAV dogs harbor a 10bp deletion in exon 9 of *COL4α5*, where as the XLAS-affected Samoyeds have a nonsense mutation in exon 35 of the same gene. Therefore, the XLAS-affected NAV dogs will have an absence of much of the collagenous domain of the COL4α5 protein, whereas the XLAS-affected Samoyeds will maintain a larger portion of the collagenous domain. The XLAS murine model mirrors the XLAS-affected NAV dogs in both the severity of the mutation and type IV collagen gene expression. This may be due to the fact that the mutation causative for XLAS in the murine model is a nonsense mutation in exon 1.

*XLAS-carrier NAV dogs*

This work also included analysis of gene expression of XLAS-carrier NAV dogs. To date, XLAS-carrier female NAV dogs have been less studied than their affected male counterparts; therefore fewer IF data are available for them and we cannot correlate gene expression changes with IF data.

Expression of *COL4α5* in XLAS-carrier females is dependent on Lyonization. Due to random X inactivation, some segments of the GBM will express the allele harboring the mutation, while others will express the wild type allele. Therefore, IF staining for COL4α5, as well as those chains that trimerize with COL4α5 (*i.e.*, COL4α3,

COL4α4, and COL4α6), is segmental (*i.e.*, mosaic) in all basement membranes in which they normally are found (*i.e.*, the GBM, BC and TBM) in young carrier females [39].

Like their affected male counterparts, carrier female NAV dogs exhibited a greater than two fold down regulation of *COL4α5* (p=0.06). Expression of *COL4α3* and *COL4α6* was also comparable to that of the XLAS-affected dogs, that is, there was no change (p=0.75 and 0.42, respectively). In direct contrast to affected males, carrier females showed a greater than two fold down regulation of *COL4α4* (p=0.03) (Figures 2 and 3). However, the observed relationship between *COL4α4* and the disease may be confounded with animal age. Although IF staining for COL4α1 and COL4α2 is consistently increased in GBM and interstitium of XLAS-carrier females [39] and although neither gene exhibited a greater than two fold change in expression (p=0.08 and 0.3, respectively) (Figures 2 and 3), the p-value of for *COL4α1* suggests a trend of down regulation.

Differences in the ages of the dogs and the stages of progression of their renal disease at time of necropsies certainly could contribute to the differences in gene expression observed in affected male and carrier female NAV dogs. The XLAS-carrier females were euthanized for reasons unrelated to ESRD and their ages varied (13 to 84 months), while XLAS-affected males were euthanized because of ESRD at a relatively young age (8 to 10 months) (Table 2).

**Table 2.** List of dogs used in study. Number, age, and gender of dogs used to evaluate type IV collagen gene expression in samples of renal cortex.

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

[a] Age of the dogs when they were necropsied and renal cortex was obtained for subsequent RNA isolation. ARHN = autosomal recessive hereditary nephropathy, XLHN = X-linked hereditary nephropathy, HN = hereditary nephropathy.

*ARAS-affected ECS*

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

Small sample size may not be the only consequence of the dogs being client owned. Because each dog was euthanized at a time determined by the owner, not by progression time points, the samples collected from ECS were taken at different time points in the disease. This fact could contribute to changes in transcript levels which may not otherwise be seen in time point matched dogs.

When compared to dogs without signs of renal disease (including normal NAVs), ARAS-affected ECS at ESRD showed a greater than two fold down regulation of *COL4α4*, similar to the down regulation of *COL4α5* seen in the XLAS-affected NAV dogs (Figures 2 and 3). The established p-value for *COL4α4* of 0.13 is not strongly significant but indicates a trend of down regulation.

*COL4α6* gene expression exhibited a greater than two fold change (Figures 2 and 3); however, a p-value of 0.36 was obtained. No provocative gene expression changes were observed for *COL4α1*, *COL4α2*, *COL4α3*, and *COL4α5* (p= 0.23, 0.48, 0.49, and 0.91, respectively) (Figures 2 and 3). The different expression changes seen for *COL4α1* and *COL4α2* in the NAV dogs versus the ECS could be explained by the fact that ECS and NAV dogs have different genetic backgrounds, and two forms of AS. This could lead one to hypothesize that different pathways for disease progression may result from for the different genetic causes of the disease.

The IF findings for ARAS-affected ECS are similar, in some respects, to those for XLAS-affected NAV dogs. Specifically, there is complete absence of COL4α3 and COL4α4, one of which likely contains the causative mutation, in the GBM and distal tubule basement membrane (dTBM) [38]. Of the two genes encoding these proteins, *COL4α4* showed a trend of down regulation and *COL4α3* showed no change in

expression. Therefore, this suggests that the mutation is present in *COL4α4*. If this is the case, the decrease in transcript levels of *COL4α4* could be explained by nonsense mediated degredation, the same would be true for *COL4α5* in the XLAS-affected NAV dogs. Unlike the NAV dogs, ARAS-affected ECS do have widespread COL4α5 staining in their renal basement membranes [38, 39]. This is due to the fact that COL4α5 can trimerize with COL4α6, and in ARAS-affected ECS, these two peptides co-localize in the BC and collecting tubule basement membranes (ctTBM), as well as to some extent in the GBM. The persistence of COL4α5 in the GBM of ARAS-affected ECS is consistent with the normal levels of *COL4α5* mRNA in these kidneys.

Currently, there exist three murine models of ARAS, two of which are *COL4α3* NC1 domain knockouts and one of which is a *COL4α3/COL4α4* transgenic insertion deleting the 5′ ends of both genes [53-55]. In those models for which northern blot or IF analysis were performed, the gene that is mutated is absent or expression is drastically reduced and this is also the case for XLAS-affected NAV dogs [53-55]. This supports our hypothesis that ARAS in the ECS is due to a mutation in *COL4α4*. However, none of the current murine models knocked out *COL4α4* alone, which together with less quantitative forms of detection, may explain the differences observed in comparison of the ECS with the *COL4α3* knock out murine model described by Miner *et al.* [54]. These differences are minor and include the increase in *COL4α1* expression in the murine model [54]. There is an increase in COL4α1 and COL4α2 staining in the interstitium of the ECS, as previously demonstrated by IF staining [38]. However, there was no significant change in expression of these genes.

## Conclusion

This report provides a comprehensive view of type IV collagen gene expression patterns in two canine models of AS. This allows for comparisons between previously published data for other murine and canine models, and also allows a comparison between two forms of AS. This study also provides a method suitable for investigating other forms of AS which have not yet been characterized, such as ADAS in the Bull Terrier [60]. Finally, the data provide evidence suggesting that *COL4α4* is the causative gene in the ECS form of ARAS. Further studies are needed to identify the causative mutation.

## Materials and methods

### *Animals*

The NAV dogs used in this study were members of a colony maintained at Texas A&M University. Status of normal and XLAS-affected NAV dogs was determined by genotype using primers designed to amplify the region harboring the 10bp deletion in *COL4α5* (Forward 5′-CGCTTGACTATTTTGTGTGTCATAA-3′, Reverse 5′-AAGGTGATGCTGTGATCTGATTTA-3′). However, the XLAS-carrier female NAV dogs were born prior to implementation of this test, and the status of these dogs was determined by IF staining of their epidermal basement membranes, as previously described [39]. XLAS-affected dogs were necropsied, whole kidneys harvested and renal cortex isolated, between eight and ten months of age, when their serum creatinine levels were ≥ 5.0 mg/dL, end-stage renal disease. Normal NAV dogs were euthanized, whole

kidneys harvested and renal cortex isolated, at eight and fifteen months of age. XLAS-carrier female NAV dogs were euthanized (for reasons unrelated to AS) and necropsied between thirteen months and seven years of age. Whole kidneys were harvested and renal cortex isolated from the XLAS-carrier females in an identical fashion to the normal and XLAS-affected NAVs. ECS used in this study were individual, client-owned dogs and were studied because they developed juvenile-onset renal failure caused by ARAS. Diagnosis of ARAS in affected ECS was confirmed by electron microscopic and IF evaluations of their GBM [38]. Affected ECS were euthanized at ESRD, between seven and nine months of age, and the renal cortex obtained. Because ECS were client-owned, kidneys from normal dogs of this breed were not available. Dogs not affected with AS, from several other breeds, were also used for this study. These dogs were both client-owned and from an unrelated research colony and included two Dalmatians, one German Shepherd Dog, one Great Dane and one Havanese (Table 2). These dogs had no signs of renal disease and were euthanized for reasons unrelated to renal function. For all dogs, renal cortex taken at the time of necropsy was stored in RNAlater (Ambion, Austin, TX, USA) until isolation of RNA.

*RNA isolation*

Total RNA was isolated from renal cortex using RNA STAT-60 (Iso-Tex Diagnostics, Inc., Friendswood, TX, USA) according to the manufacturer's protocol. The resulting RNA was cleaned using the Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) and the RNase-Free DNase Set (Qiagen Inc.), to avoid DNA contamination. RNA quality was assessed by one step RT-PCR with β-actin primers (Forward 5′-

TGCGTGACATTAAGGAGAAG-3′, Reverse 5′-CTGCATCCTGTCGGCAATG-3′), using the Sigma Enhanced Avian HS RT-PCR kit (Sigma Aldrich, St. Louis, MO, USA). RNA was quantified by spectrophotometry and diluted to 1ng/ul.

*Real time quantitative RT-PCR*

The mRNA levels for the six type IV collagens and *GAPDH* (used for normalization) were quantified on the BioRad MyiQ Single-Color Real-Time PCR Detection System (BioRad Inc., Hercules, CA, USA). Primers and probes (Table 3) were based on the published canine sequence and designed using Primer Express 1.0 (Applied Biosystems, Foster City, CA, USA). When possible, probes were designed to span two exons to eliminate amplification of any DNA that might still be present. The 5′ and 3′ ends of the probes were labeled with 6-FAM (6-carboxyfluorescein) and Black Hole Quencher™, respectively (Biosearch Technologies, Novato, CA, USA). Each 25ul reaction contained 0.2uM of each forward and reverse primer and probe, 12.5ul of 2x QuantiTect Probe RT-PCR Master Mix (Qiagen Inc.), 0.25ul of QuantiTect RT Mix (Qiagen Inc.) and 7ng of RNA. The amplification conditions were: 50° for 30 seconds, 95° for 13 minutes and 30 seconds followed by 45 cycles at 95° for 10 seconds, 55° for 30 seconds and 70° for 30 seconds. In order it ensure reproducibility, each sample was amplified at least in duplicate.

**Table 3.** Primer and probe sequences for quantitative real time RT-PCR.

| Name | Size (bp) | Sequence 5′-3′ |
|---|---|---|
| COL4A*3* | 74 | Forward-GAGCCTTATATTAGCAGATGCAC<br>Reverse-TCAGTGGTTTGGCTGTGAATG<br>Probe-TGTGAAGGTCCTACGATTGCCATAG |
| COL4A*4* | 70 | Forward-GGCTACAGTCTGTTATACCTGAAGGA<br>Reverse-AAGACCCCTGCCAGACCAA<br>Probe-AGGAGAAGGCCCACAATCAGGA |
| COL4A*5* | 170 | Forward-GAGCATGGAGCCCCTGAA<br>Reverse-TCGTGTGCATCATGAAGGAATAG<br>Probe-CCAGAGCATCCAGCCATTCATTAG |

*Analysis*

In order to obtain fold changes, analysis was carried out as described by Pfaffl [69] assuming a PCR efficiency (*E*) equal to two. All affected and carrier dogs were compared with dogs exhibiting no renal disease (Figure 4). Fold changes greater than two were considered to be indicative of changes in gene expression. P-values were calculated for each group of dogs using the bootstrap with replacement method [70].

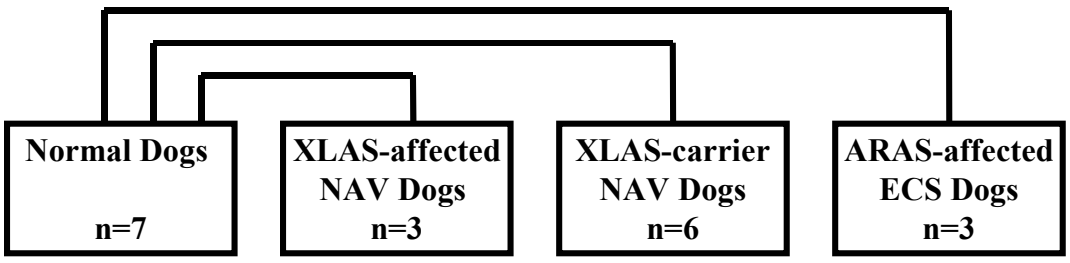


**Figure 4.** Comparisons made to assess gene expression patterns in kidneys of dogs with AS.

# CHAPTER III

# GENETIC CAUSE OF AUTOSOMAL RECESSIVE HEREDITARY NEPHROPATHY IN ENGLISH COCKER SPANIELS*

## Background

Although terms used to identify the condition have varied over the years, a hereditary renal disease has been observed in the ECS since the late 1940s. Krook described a juvenile nephropathy that he termed renal cortical hypoplasia in 40 Cocker Spaniels that were examined at the Royal Veterinary College in Stockholm from 1946 to 1956 [59]. He determined that the condition was characterized by albuminuria [59]. Additional early reports about renal cortical hypoplasia in Cocker Spaniels from Sweden, Switzerland and Australia also described juvenile onset of a fatal proteinuric nephropathy [71-73]. Later studies demonstrated that the disease was inherited in an autosomal recessive fashion and that the primary renal lesions involved glomeruli [74-77]. With these developments, familial nephropathy (FN) became the diagnostic term most widely used for the disease. We believe that hereditary nephropathy is the most appropriate diagnostic term for this type of primary glomerular disease in dogs.

Beginning in the 1980s, investigators using transmission electron microscopy noted that the ultrastructural appearance of the glomerular basement membranes (GBM) of affected dogs resembled that of the GBM in human AS [78, 79], but that morphologic finding was of little help while the molecular and genetic basis of AS remained obscure.

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In the late 1980s a series of discoveries established that AS was caused by type IV collagen defects [30, 44, 63, 66]. Thus, when distinctive ultrastructural GBM changes were identified in an affected dog examined in 1992 at Texas A&M University [80], systematic investigations of the disease based on the hypothesis that it was a type IV collagen disorder were initiated. Subsequent studies characterized the clinical, pathologic, and ultrastructural features of the disease, including the pattern of abnormalities observed as the disease progressed [38, 81]. Importantly, immunostaining of kidney from affected dogs demonstrated complete absence of the COL4α3 and COL4α4 peptide chains that normally are present in the GBM [38]. This finding further suggested that the mutation responsible for the disease was in one of the two genes encoding these proteins.

Each of six type IV collagen genes encodes a distinct α chain (1-6) that can be assembled into three heterotrimers: α1.α1.α2, α3.α4.α5, and α5.α5.α6 [30]. In adult kidney, the collagen network that provides the structural framework for the GBM is composed of α3.α4.α5 heterotrimers. This network is essential for normal GBM structure and function because renal disease, most notably AS, ensues when it is absent or abnormal [30, 50]. Specifically, mutations in any one of the three genes encoding the COL4α3, COL4α4, or COL4α5 proteins can cause AS because a defect in any of the three chains can prevent proper assembly. The *COL4α5* gene, which encodes the COL4α5 chain, is located on the X-chromosome in humans and dogs [15, 30], and mutations in this gene cause human X-linked AS and canine X-linked hereditary nephropathy (XLHN) [30, 40, 58, 82]. The *COL4α3* and *COL4α4* genes, encoding the COL4α3 and COL4α4 chains, respectively, are located on human chromosome 2 and canine chromosome 25 [15, 30]. Mutations in either one of these two genes cause

autosomal forms of AS in humans, and the same is suspected to be true of autosomal forms of canine hereditary nephropathy [44, 46, 47, 66, 83-85].

In this work the candidate gene approach was used to identify the genetic cause of autosomal recessive hereditary nephropathy (ARHN) in the ECS. Sequencing of COL4A*4* identified a single base change in exon 3 that produces a premature stop codon. This is the first autosomal form of canine hereditary nephropathy for which the underlying genetic cause has been identified. Appropriate use of a genetic test for this mutation will permit breeders of ECS to eradicate the disease.

**Results**

In total, all or part of COL4A*4* was sequenced in genomic DNA samples obtained from 134 ECS. These subjects were 12 affected dogs, 8 obligate carriers, and 89 other dogs from the United States and Canada, as well as 19 dogs from Great Britain and 6 dogs from Sweden.

To identify the causative mutation, the coding and the flanking intronic regions of COL4A*4* was sequenced and analyzed in its entirety for two affected dogs and two obligate carriers. The sequence obtained was compared with that obtained from two ECS of unknown status and two normal mixed breed dogs, as well as with the published canine coding sequence for *COL4α4* (accession AY2633363) and the published 7X NIH genomic reference sequence for the canine. This analysis revealed a single nucleotide substitution (adenine changed to thymine) at base 115, causing a nonsense mutation (lysine changed to a stop codon) in exon 3 of both affected dogs (Figures 5 and 6). Additionally, each of the two carriers had both an adenine and a thymine (*i.e.*, an adenine

from the normal allele and a thymine from the mutated allele) at base 115 (Figures 5 and 6).

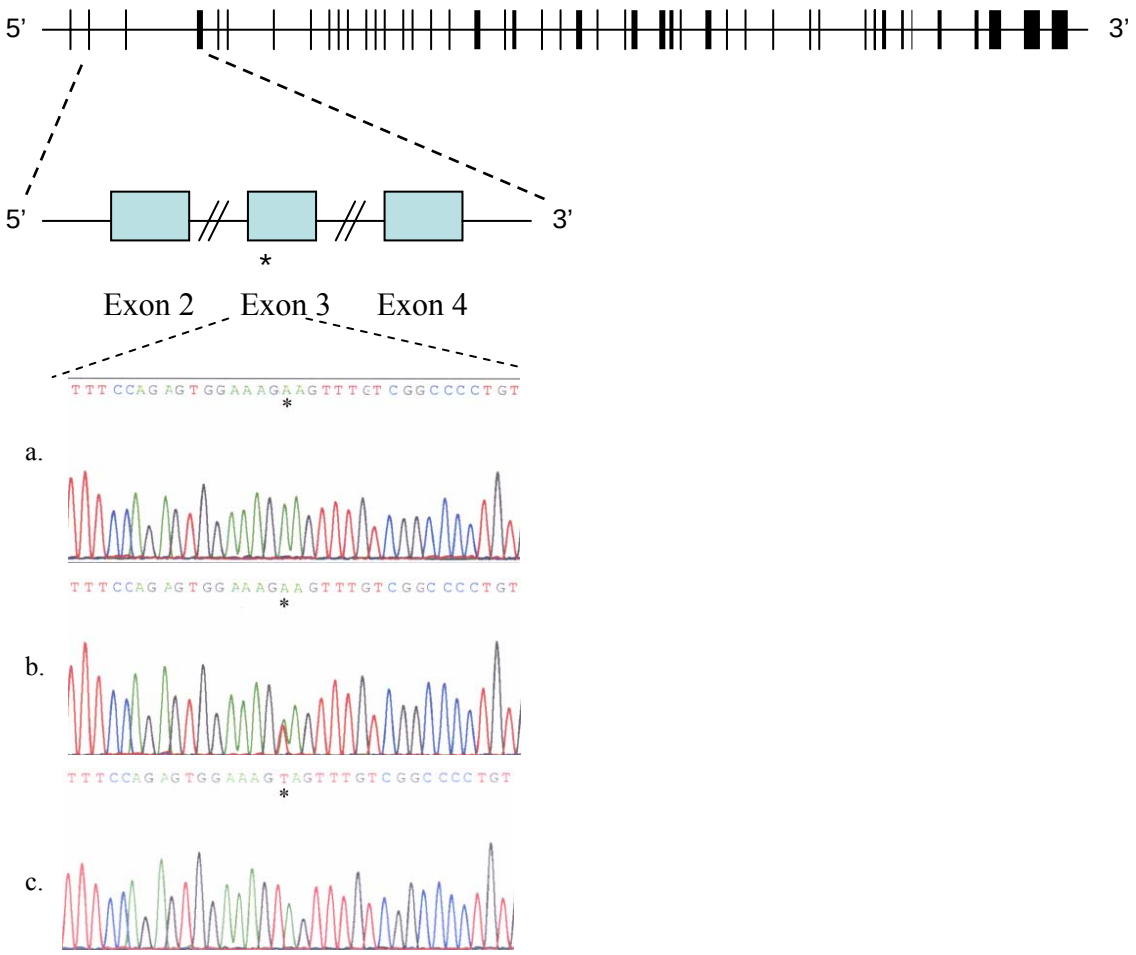


**Figure 5.** Schematic representation of genomic *COL4α4* (top), exons 2, 3 and 4 (enlarged beneath), and chromatograph (bottom). A star represents the location of the nonsense mutation. Chromatograph shows nucleotide sequences for ECS that: a) does not have the mutated allele that causes ARHN, b) is a heterozygous carrier of the mutated allele, and c) is a dog affected with ARHN because it is homozygous for the mutated allele. Asterisks indicate the position of the single base change in each dog's nucleotide sequence.

Normal

S G K K F V G F V G P C G G R D C
AGTGGAAAGAAGTTTGTCGGCCCCTGTGGAGGAAGAGATTGCTCGGTGTGC
AGTGGAAAGTAGTTTGTCGGCCCCTGTGGAGGAAGAGATTGCTCGGTGTGC
S G K *

Affected

**Figure 6.** Nucleotide and amino acid sequence in exon 3 (base 106 – 157). Sequence for normal ECS is shown on top in black, and sequence for affected ECS is shown on bottom in red. The nonsense mutation is boxed, and truncation is indicated by a star.

To verify the mutation, exon 3 of *COL4α4* was sequenced using genomic DNA from 12 ECS in which ARHN had been diagnosed, as well as from 8 obligate carriers. In every instance in which an affected or obligate carrier dog's DNA was sequenced and analyzed, the same mutation was identified (*i.e.*, homozygous in the affected dogs, and heterozygous in the carriers).

Sequence analysis of *COL4α4* identified two single nucleotide polymorphisms SNPs) that also co-segregated with the disease. Both of these SNPs are in the intron upstream of exon 42. The first, which is a thymine to adenine substitution, is located 93 bases upstream, while the second, which is a thymine to cytosine substitution, is 90 bases upstream. These two nucleotide changes do not alter the coding sequence of the gene, but they are inherited with the disease.

Exon 3 of *COL4α4* was also sequenced using genomic DNA from 114 ECS having unknown genetic status. These dogs included 65 that were close relatives of obligate carriers (*i.e.*, parents, siblings, or offspring) in which the random odds of finding the dog to be heterozygous for the mutation were one in two (*i.e.*, 50% chance of also

being a carrier). Of these 65, 34 dogs (52.3%) were heterozygous for the mutation, and therefore, carriers. Of the remaining 44 dogs, in which the chances of being a carrier were ≤ 25%, three (6.8%) were heterozygous for the mutation. Five dogs (two of which were identified as carriers) could not be placed into the above categories due to the absence of relationship information or conclusive diagnosis. Therefore, a total of 39 previously unknown carriers were identified.

Light microscopic examination of stained sections of formalin-fixed, paraffin-embedded kidney from the Swedish ECS that died of juvenile-onset renal failure was sufficient to exclude ARHN as the cause of that dog's renal disease. The histologic lesions were unmistakably those of renal dysplasia. Samples of DNA were available from the affected dog, both of its parents, and three other closely related dogs. Tests for the mutated *COL4α4* allele that causes ARHN were negative in these six dogs.

Results of the pathologic evaluations of kidney from the British ECS that was suspected to have ARHN were inconclusive. The light microscopic and transmission electron microscopic findings were compatible with the disease, but they were not sufficiently distinctive to permit definitive diagnosis. Glomerular ultrastructure was too poorly preserved in the available formalin-fixed tissue for critical evaluation, and the material was not suitable for immunostaining. However, DNA specimens were available from six related dogs, including the dog's sire. Genetic testing demonstrated that the sire was heterozygous for the mutated *COL4α4* allele and that the other five dogs were homozygous for the normal allele.

Overall, the sequence data we obtained for exon 3 of *COL4α4* in ECS identified the genetic status of 134 dogs from the United States, Canada, Great Britain, and Sweden.

With 12 affected dogs and 47 carriers in this sample, the overall frequency of the mutated allele in the study population was 0.2649 (71 of 268). However, this sample included at least four separate kindred, and in two small kindred (containing 19 and 6 dogs) in which an affected dog had not been diagnosed, the frequencies of the mutated allele were 0.0263 (one of 38) and 0 (none of 12), respectively. This frequency, however, is not an accurate representation for the ECS population as a whole because the four kindred studied were selected based on the incidence of AS in their line.

**Discussion**

Predictable consequences of the missense mutation in exon 3 of *COL4α4* explain all of the salient features of ARHN in affected ECS. One feature of ARHN is that levels of mRNA for *COL4α4* are greatly reduced in kidney of affected dogs as assessed by qRT-PCR, but levels of transcripts for *COL4α3* and *COL4α5* are not reduced. A similar pattern of altered mRNA levels has also been observed in dogs and mice with missense *COL4α5* mutations in which mRNA for that gene is reduced, but mRNA for *COL4α3* and *COL4α4* are not reduced [52]. The mechanism by which such mutations lead to reduced levels of mRNA for their respective genes is uncertain, but nonsense-mediated decay is a potential explanation [86]. A second feature of ARHN in affected ECS is that immunostaining demonstrates total absence of COL4α4 chains and COL4α3 chains in GBM, together with greatly reduced GBM COL4α5 chain expression [38]. Because the mutation creates a premature stop codon, any protein synthesis directed by the mutated gene would result in a severely truncated peptide that would be unable to combine with its normal partners (*i.e.*, COL4α3 and COL4α5 chains) to produce stable α3.α4.α5

heterotrimers. Without the ability to form α3.α4.α5 heterotrimers, the only collagen networks that can form in renal basement membranes of ECS with ARHN are composed of α1.α1.α2 or α5.α5.α6 heterotrimers, or both. This accounts for the observation that all renal basement membranes in ECS with ARHN that exhibit COL4α5 chain expression by immunostaining also exhibit co-expression of COL4α6 chains, but renal expression of both COL4α3 chains and COL4α4 chains are totally absent even where COL4α5 chains are expressed [38]. Finally, absence of the α3.α4.α5 network in the GBM of affected dogs accounts for all of the ultrastructural, clinical, and pathologic features of the progressive nephropathy that occurs in ECS with ARHN. The nephropathy that develops in these dogs has essentially the same ultrastructural, clinical, and pathologic features as the nephropathy that occurs in dogs with X-linked hereditary nephropathy in which the α3.α4.α5 network is missing from the GBM because of a *COL4α5* mutation [39, 50, 57, 87, 88].

The fact that ARHN has persisted in the ECS breed for more than 50 years is a testament to the difficulties that have previously existed in identifying carriers of the mutated allele. In the past, carriers have been accurately identified only when a mating produced at least one affected dog that was properly diagnosed. Given that the random odds of any single dog produced by a carrier to carrier mating being affected are only one in four (25%) and that proper diagnosis of ARHN required very specialized evaluations, carriers were not always identified even when they were mated to one another. Additionally, all that was necessary to avoid risk of producing a dog that might develop juvenile onset renal failure caused by ARHN was to select pairs for mating such that at least one of the two dogs was highly unlikely to carry the mutated allele. However, for

any mating of a carrier to a genetically normal dog, each offspring's random odds of being a carrier are one in two (50%). Thus, although astute breeders commonly managed to avoid producing affected dogs, the mutated allele readily persisted in the gene pool and unsuspected carriers eventually were mated to one another from time to time. Moreover, each diagnosis of ARHN typically had a devastating effect on any line of ECS in which it was discovered. Not only were the obligate carriers no longer bred, but all breeding to closely related dogs was frequently halted because of uncertainty about their carrier status for ARHN, regardless of their other desirable traits. Availability of a test for the mutated *COL4α4* allele should solve these problems.

In ECS with renal disease, a test for the mutated *COL4α4* allele will be useful only in those dogs with clinical and light microscopic findings that are compatible with a diagnosis of ARHN. Testing all ECS with any type of renal disease for the mutated allele will not be helpful. Moreover, even in young ECS with renal disease, testing for the mutated allele should not substitute for a through clinical and pathologic investigation of the dog's illness for the following reason. A genetic test that excludes ARHN provides no information about what disease actually caused the nephropathy. That is, absent an appropriate clinical and pathologic investigation, the cause of the dog's renal disease is certain to remain obscure in such an instance.

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

about the correct diagnosis. Conversely, pathologic features of the nephropathy in the dog from Sweden were sufficient to exclude ARHN as its cause, and as expected, tests for the mutated allele in dogs from that kindred were negative. Nevertheless, results of genetic testing were not required to make the diagnosis, which was instead based on the results of clinical and histopathologic evaluations.

A test for the mutated *COL4α4* allele has obvious utility as an aid in the diagnosis of ARHN in the ECS with renal disease. However, it will be of greatest value in guiding future selective breeding of these dogs. Indeed, proper use of a test to detect the mutated allele should permit breeders of ECS to eradicate ARHN from this breed in as short a time as one generation and without having to abandon any breeding lines that have other desirable traits. First, it will be possible to always know before a dog is bred whether or not it carries the mutated allele. Obligate carriers need not be mated at all unless there is a compelling reason; for example, to maintain some other highly desirable trait in the line. If breeding a carrier is judged to be potentially important, the first step is to verify by testing that the other dog in the pair does not carry the mutated allele. This avoids all risk of the mating producing a dog that will develop ARHN. The second step is to also test the progeny of the mating. Some progeny will be carriers (the random odds for each dog are one in two); however, such carriers can be confidently placed with owners who are seeking a pet because the health of these dogs is the same as that for genetically normal dogs. In addition, breeders can select among the progeny that are not carriers to choose the animals to keep as breeding stock and thus maintain the line's most desirable traits.

## Materials and methods

*Dogs and samples*

From 1993 through 2005, ARHN was diagnosed at Texas A&M University in 18 ECS. Each diagnosis was based on finding both the characteristic ultrastructural GBM changes by transmission electron microscopy and the distinctively abnormal pattern of type IV collagen expression in renal basement membranes by immunostaining, as previously described. Diagnosis of ARHN in individual dogs identified specific families in which the disease was inherited, so family histories were collected. Parents of affected dogs were presumed to be obligate heterozygous carriers of the disease trait. Whenever possible, genomic DNA was obtained from affected dogs, each of their parents, and as many closely related dogs as were available. Additionally, DNA samples were collected from some ECS that were not known to be related to any dog in which ARHN had been diagnosed.

Additionally, during 2005 and 2006, formalin-fixed specimens of kidney from ECS with juvenile-onset renal failure were received from Great Britain (two dogs) and Sweden (one dog). Before shipment to Texas A&M University, the tissue samples were processed and embedded for routine light microscopic and transmission electron microscopic evaluations (performed at the Institute of Pathology and Genetics in Loverval, Belgium). Upon receipt, the embedded tissues were sectioned, stained, and examined by standard methods, as previously described [38]. Family histories were also collected for these dogs, together with DNA specimens from closely related dogs.

Puregene DNA Isolation Kit was used to isolate DNA (Gentra Systems, Minneapolis, MN) from nucleated cells in whole blood or buccal swab specimens, according to the manufacturer's instructions.

*Sequencing of COL4α4*

Results from previous studies, reported elsewhere, demonstrated that mRNA transcripts for *COL4α4*, but not for *COL4α3* or *COL4α5*, were reduced greater than 2-fold, as measured by a quantitative real-time polymerase chain reaction (qRT-PCR) assay, in kidney of ECS with ARHN (described in chapter II). This pattern was similar to the reduction of *COL4α5* mRNA that was observed in dogs with X-linked hereditary nephropathy, known to be caused by a deletion in *COL4α5*. Based on this observation, we hypothesized that the cause of ARHN in the ECS was most likely a *COL4α4* defect. Thus, that gene was sequenced.

The canine *COL4α4* gene contains 47 exons, and the entire coding region was sequenced and analyzed. Most of the gene (41 exons) was sequenced by amplifying individual exons together with their flanking intronic sequences and then sequencing each exon separately. However, three pairs of exons (10 and 11, 13 and 14, and 38 and 39) were sequenced together because of the short length of their intervening introns. Thus, a total of (45) sets of primers were designed to amplify portions of COL4α4 using the published canine genome sequence (Table 4). Intron/exon boundaries were determined by aligning the canine genome sequence in the region on chromosome 25 around *COL4α4* and the previously published mRNA sequence for *COL4α4* (accession No. AY263363).

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

To identify the mutation, sequence was obtained from two affected dogs, two obligate carriers, two ECS of unknown genetic status, and two normal mixed breed dogs. Sequences were aligned using Clustal W (http://www.ebi.ac.uk/clustalw/) and nucleotide polymorphisms that segregated with ARHN in the ECS were identified. To verify the mutation, exon 3 was amplified and sequenced in every affected ECS and obligate carrier from which an adequate DNA sample was available.

Once the causative mutation was identified, exon 3 also was amplified and sequenced in 114 additional ECS having unknown genetic status. This was accomplished using the same primers for exon 3 (Table 4) and nucleotide sequencing procedure as has already been described.

**Table 4.** Primers and melting temperatures ($^{o}$C) used for PCR amplification of canine *COL4α4*

| Exon | Tm | Sequence |
|---|---|---|
| 1 | 55 | F: CAGGGCATAGAACCTCACTTA |
| | | R: CTGCTGTGCTCTGGACATTAG |
| 2 | 55 | F: TCACTAATGACAGCAGCCTAT |
| | | R: ACCTGGGTAACTTGGTAAGAA |
| 3 | 55 | F: CCCTCTCACCAAGCCAC |
| | | R: GTTGCTGACTGTTGTTAGATGTT |
| 4 | 55 | F: GTTTGTGTTAGAAGAGAGCG |
| | | R:CATAGTAGTGCTGGTGAGTGG |
| 5 | 55 | F: GCTCATTATTTATGTTTTCAAG |
| | | R: AAGCACAGTAGGGAGAGGG |
| 6 | 57 | F: GAGTCACCAT TGCCATAACG |
| | | R: CAGCCTCCTCCCACAGTCT |
| 7 | 55 | F: GAAATCTCCACTAGCGAAAC |
| | | R: GCAAGAACAGTTAGGAGATACT |
| 8 | 57 | F: CCACACAGCCTTCCACAGTT |
| | | R: ACCCAGGTAATGCCAAATGAT |
| 9 | 55 | F: GATGTTTCTGGGACTGTGAT |
| | | R: ACTGGTAATGGGAGGTGTA |
| 10 and 11 | 55 | F: GAACCCAGGGCAACC |
| | | R: TTAACATCTGCTCCTCCAT |
| 12 | 55 | F: GCCACGCAGGATTGTATG |
| | | R: GCTGAGGTTGCTTTGGG |
| 13 and 14 | 55 | F: GAAGAGATAATGTCTGAAAGATGTA |
| | | R: CCCAGGTGCCCCAATA |
| 15 | 55 | F: GCCATAAAGCAGTTTCATAAG |
| | | R: ATCTGTAAAATAAATGTGTCTCC |
| 16 | 55 | F: ATGCGATACTGAGATTTTGC |
| | | R: GATACGAGGTGATCCCCA |
| 17 | 55 | F: GTCGGATTCCTTTGTCATTC |
| | | R: CCACCCAAGTCCCATCTC |
| 18 | 55 | F: CAGTGCTGCTCCAAGTTC |
| | | R: GGTGAGGGTGAGGCTGTC |
| 19 | 55 | F: CGGTTTCCATTTGTGTGC |
| | | R: CAGGCTTCATAGAACTGTTTG |
| 20 | 55 | F: CTTAGAGAGAAAGAGTCATAGGAA |
| | | R: AGGAGTGCTCATAGGCGTA |
| 21 | 55 | F: CCCCCCAACAGACCAT |
| | | R: CAGCACTGAGAACAGCACC |

**Table 4.** Continued

| Exon | Tm | Sequence |
|---|---|---|
| 22 | 55 | F: AGGTCAAGAGCCTCAGTTTTAT |
| | | R: GAAATGTGAACAGCAAGGAATA |
| 23 | 55 | F: GTCCTGTGTTTCCTCCTACT |
| | | R: CCAAAGATGGCTCTGATTA |
| 24 | 55 | F: GGTTTGCTATTGAGTAACTGTCTA |
| | | R: TTATTGAACGGTTCTGCTGTA |
| 25 | 55 | F: AGGCAGTTCAAATCGTCTC |
| | | R: AACTATTGGTTCATCATCTTAC |
| 26 | 55 | F: AGGCGAGGCAACAGTTACATA |
| | | R: CCCTGGACCACCTGCTTAC |
| 27 | 55 | F: CAAGGTGGCAAAGCAAC |
| | | R: GCATTCTACATTTCTAAGGC |
| 28 | 55 | F: CGTCGGTTGCTGGTACT |
| | | R: GCTACTTGTCATTCTGTGGAG |
| 29 | 55 | F: GATGGATGTTGCTTCGTG |
| | | R: GGATGGACAGTATCAGGCT |
| 30 | 55 | F: GTCCCACATCAGACTTCCT |
| | | R: CTAAAGCAGACACCAGCAA |
| 31 | 55 | F: TACTGTGCTGATACTGTGCTG |
| | | R: GCTGGAACTGGTATTAGATGT |
| 32 | 55 | F: TATGGCTTAGGGCAGGAA |
| | | R: AAGGGCAATGATGTTTACAGA |
| 33 | 55 | F: CACCTCTAATACTGGAGTTGTA |
| | | R: ATGCTAAATGTGCGTGCT |
| 34 | 55 | F: TGAAGATAAACTATAAAGACAAAT |
| | | R: TGGAGCCCAACACAAG |
| 35 | 55 | F: CAAGGGCTGAAGTTGGAGGTT |
| | | R: GAGGGATGGGTAGGTCTGAGTG |
| 36 | 55 | F: AGGTCTAGGGGATAAAAGTG |
| | | R: CTGAGTGAGAGAGAAGAGGAA |
| 37 | 55 | F: GAGCGTGTTAAATAATAGCCA |
| | | R: TCATCTTCAGTCCTAATAATAGTCC |
| 38 and 39 | 55 | F: GCAGCAGGTGGTTGGTCTCAGCA |
| | | R: CCACCTGCCGCATTGA |
| 40 | 55 | F: CCCTTCATCTCTCGCTTGC |
| | | R: GAACTTGTGTTTCTTCCCCTTAC |
| 41 | 55 | F: TGGGTTCAGTCCATCAGA |
| | | R: GAATAGGGTCCTCACATACAG |
| 42 | 55 | F: GGATGGGGACTTAGTTATGTA |
| | | R:  AAGCACTCACGCTCTGG |
| 43 | 55 | F: GGACTGTTGAGCATTCTTTG |
| | | R: GCTTACACTGCCCCATACT |
| 44 | 58 | F:  CTCGGGCTCAGGGTCTAAC |
| | | R: GGCTGCGGATCAGTGC |
| 45 | 55 | F: CTCCTCCTCTCTGGCTCC |
| | | R: TAAAATGTTGATGAATCTGTAAAAT |
| 46 | 56 | F: GGAGGCGTGTCTGTGGGT |
| | | R: CCGTGTCTCAAGAGGCTATGG |
| 47 | 55 | F: GTTGGTTCTTCCCTGGATAAT |
| | | R: AACTGGAGTCTGAAATGAGCAC |
| 25 | 55 | F: AGGCAGTTCAAATCGTCTC |
| | | R: AACTATTGGTTCATCATCTTAC |

**Table 4.** Continued

| Exon | Tm | Sequence |
|---|---|---|
| 26 | 55 | F: AGGCGAGGCAACAGTTACATA |
| | | R: CCCTGGACCACCTGCTTAC |
| 27 | 55 | F: CAAGGTGGCAAAGCAAC |
| | | R: GCATTCTACATTTCTAAGGC |
| 28 | 55 | F: CGTCGGTTGCTGGTACT |
| | | R: GCTACTTGTCATTCTGTGGAG |
| 29 | 55 | F: GATGGATGTTGCTTCGTG |
| | | R: GGATGGACAGTATCAGGCT |
| 30 | 55 | F: GTCCCACATCAGACTTCCT |
| | | R: CTAAAGCAGACACCAGCAA |
| 31 | 55 | F: TACTGTGCTGATACTGTGCTG |
| | | R: GCTGGAACTGGTATTAGATGT |
| 32 | 55 | F: TATGGCTTAGGGCAGGAA |
| | | R: AAGGGCAATGATGTTTACAGA |
| 33 | 55 | F: CACCTCTAATACTGGAGTTGTA |
| | | R: ATGCTAAATGTGCGTGCT |
| 34 | 55 | F: TGAAGATAAACTATAAAGACAAAT |
| | | R: TGGAGCCCAACACAAG |
| 35 | 55 | F: CAAGGGCTGAAGTTGGAGGTT |
| | | R: GAGGGATGGGTAGGTCTGAGTG |
| 36 | 55 | F: AGGTCTAGGGGATAAAAGTG |
| | | R: CTGAGTGAGAGAGAAGAGGAA |
| 37 | 55 | F: GAGCGTGTTAAATAATAGCCA |
| | | R: TCATCTTCAGTCCTAATAATAGTCC |
| 38 and 39 | 55 | F: GCAGCAGGTGGTTGGTCTCAGCA |
| | | R: CCACCTGCCGCATTGA |
| 40 | 55 | F: CCCTTCATCTCTCGCTTGC |
| | | R: GAACTTGTGTTTCTTCCCCTTAC |
| 41 | 55 | F: TGGGTTCAGTCCATCAGA |
| | | R: GAATAGGGTCCTCACATACAG |
| 42 | 55 | F: GGATGGGGACTTAGTTATGTA |
| | | R: AAGCACTCACGCTCTGG |
| 43 | 55 | F: GGACTGTTGAGCATTCTTTG |
| | | R: GCTTACACTGCCCCATACT |
| 44 | 58 | F: CTCGGGCTCAGGGTCTAAC |
| | | R: GGCTGCGGATCAGTGC |
| 45 | 55 | F: CTCCTCCTCTCTGGCTCC |
| | | R: TAAAATGTTGATGAATCTGTAAAAT |
| 46 | 56 | F: GGAGGCGTGTCTGTGGGT |
| | | R: CCGTGTCTCAAGAGGCTATGG |
| 47 | 55 | F: GTTGGTTCTTCCCTGGATAAT |
| | | R: AACTGGAGTCTGAAATGAGCAC |

# CHAPTER IV

# ISOLATION, CULTURE AND CHARACTERIZATION OF CANINE SERTOLI CELLS

## Background

Collagen proteins play a significant role in cell and tissue structure as well as cell differentiation, growth, and adhesion. Specifically, the type IV collagens are critical structural components of basement membranes throughout the body [14, 33, 89]. Despite their great functional importance and the ubiquitous presence of basement membranes, regulation of this gene family is incompletely understood. It is known that the six type IV collagen genes are unique in their genomic arrangement and expression patterns. Each gene pair shares a single promoter region and is situated in a head-to-head formation on three separate chromosomes. *COL4α1*-*COL4α2*, *COL4α3*-*COL4α4*, and *COL4α5*-*COL4α6* are transcribed from opposite strands on CFA 22, 25, and X, respectively [15, 35]. These genes, therefore, would appear to have the potential to be expressed in a straightforward manner, *i.e*. each pair, sharing a promoter, expressed together. However, the functionality of these gene products is dependant upon their ability to form three distinct heterotrimers: α1.α1.α2, α3.α4.α5, and α5.α5.α6 [34]. This leads to the tissue-specific manner in which one such pair, *COL4α5* and *COL4α6*, is expressed [11, 33, 35, 90].

The few studies done to characterize the regulation of type IV collagen genes have typically been carried out in either immortalized cell lines or renal tissue [91-95]. The use of immortalized cell lines, however, does not accurately represent *in vivo*

regulation of these genes. Renal tissue has been an attractive model for these studies because it is one of the few organs which is relatively accessible and also expresses all of the type IV collagens. Unfortunately, they are not all expressed within the same basement membrane. The α1.α1.α2 trimer is found in all basement membranes, but the remainder of the type IV collagen genes are expressed in a tissue-specific fashion. The trimer composed of α3.α4.α5 is found in the glomerular basement membrane, while that composed of α5.α5.α6 is found in Bowman's capsule [11, 33, 36, 37]. While the kidney may be a powerful system to study these genes, it is certainly not ideal. An ideal system would express all six chains in one basement membrane, from one cell type, as well as be easily accessible. These criteria are met only by one cell type in the body, Sertoli cells. The seminiferous tubule basement membrane (STBM), laid down by Sertoli cells in the testes, contains all three trimers made by type IV collagen genes [12]. This cell type is also readily available in the form of testes from castrated animals, such as the dog. Harvey *et al.* recently utilized frozen sections of testes removed from castrated dogs to study the expression of type IV collagens, demonstrating the usefulness of this tissue type [96]. Similar studies would benefit from using not only the frozen sections of such tissues, but also from the use of a primary cell culture model.

The dog is certainly and excellent model system to study human diseases, including but certainly not limited to diseases involving several types of collagens, such as AS and dystrophic bullous epidermolysis [38, 39, 58, 97]. From these disease-related studies comes not only important information regarding the disease, such as its cause, progression or treatment, but also functional information about the disease process, proving that the dog can serve as a model for understanding physiological and cellular

functions as well. Aside from being used as a model system, interest in canine research has grown to areas in which the primary focus is information directly significant to the dog.

Primary Sertoli cell cultures have been established for other animals including the sheep and rhesus monkey, but not the dog [98, 99]. This study sought to establish a method to isolate and culture Sertoli cells from dogs following castration. The methods described here can be used to establish a system to study not only functions typically associated with Sertoli cells and the male reproductive system, but as described above, to study the expression of type IV collagens as well. Our system will be unique in that other studies involving Sertoli cells have focused on the function of these cells, specifically spermatogenesis and the genetic regulation of these functions while we propose the use of this system as an *in vitro* model to study type IV collagens [98-101].

## Results

### *Isolation and culture of Sertoli cells*

Sertoli cells were isolated from testes of castrated dogs through physical and enzymatic digestion. Once the cells attached to the flask/plate they were easily subcultured or frozen for later use. Cultures were grown to confluency, producing a monolayer with a consistent morphology indicating a mostly homogenous culture. This morphology was maintained throughout each passage and after thawing from frozen stocks. Cultured cells sustained a steady growth curve for several passages, dividing to confluency for seven passages, at which point division decreased dramatically. Confluency was typically reached by two days for earlier passages and could take as long

as ten days for later passages. Cells at passage one and passage seven (final passage) are shown in figure 7.

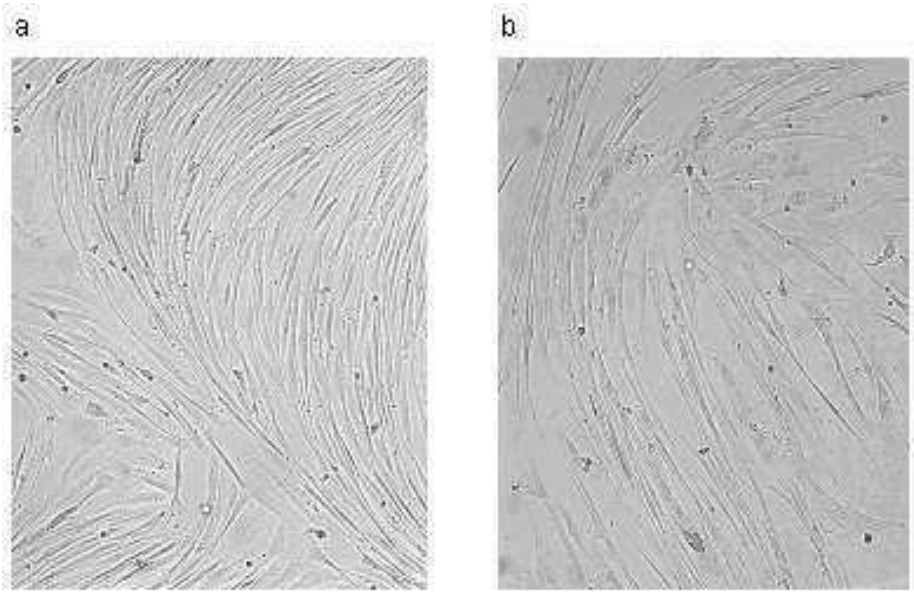


**Figure 7.** Pictures depicting morphology of isolated cells. a) cells at passage one and b) cells at passage seven.

*Characterization of Sertoli cells*

Cells were positively identified as Sertoli cells through western blot and sequence analysis. Western blot analysis was performed for both SOX9 and Clusterin B. Sertoli cells are the only cell type in the testes which produce these proteins; therefore, positive staining for these two proteins excluded other cell types potentially isolated and confirmed the presence of Sertoli cells. Figure 8 shows the western blot images: note the unique staining pattern and size rage for Cluterin B. This is due to the glycosylation of this protein. SOX9 did not have the expected size. Rather, staining was of a band twice the size expected for this protein. This is indicative of the presence of a multimer, which

would cause antibody staining at higher molecular weights, in multiple increments of the protein's molecular weight.

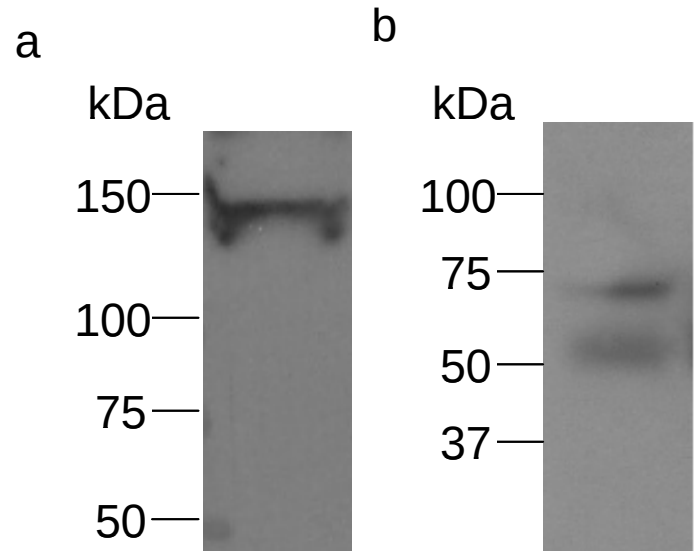


**Figure 8.** Western blots. These show antibody staining of a) SOX9 dimer (~136 kDa); note the lack of staining of the monomer (~68 kD) and b) Clusterin B (43-64 kDa).

Because the western blot results for SOX9 did not show binding to the protein of the correct molecular weight, sequence verification was performed to confirm the presence of the *SOX9* transcript. Therefore, cDNA isolated from the cells in culture was amplified, and a product at the expected size (determined by gel electrophoresis) was gel extracted and sequenced. Sequence alignment of the obtained sequence to the known sequence of canine *SOX9* verified the presence of the *SOX9* transcript, thereby, providing evidence to support the idea that the protein detected by western blotting is indeed a SOX9 multimer.

Figure 7 shows the morphology of the isolated canine cells. Along with the consistency of the morphology, the cells also show a strong similarity to the Sertoli cell lines already published [98, 99, 102]. This similarity, along with the western blot results, verified by sequence analysis when necessary, for both proteins known to be produced in Sertoli cells, demonstrate the cells isolated and cultured were indeed Sertoli cells.

**Conclusions**

Primary culture of Sertoli cells will allow for further study into areas typically associated with this fundamental cell type of the testes, as well as allow for study into areas perhaps not initially associated with these cells. Because Sertoli cells are one of a select set of cell types which express all six genes in the type IV collagen family (alpha 1-6), and because these cell types are easily accessible, they are an ideal candidate for the study of the expression of these genes [12].

This cell system can also allow for further study of Sertoli cells in general. Recent research has implicated Sertoli cells as harboring potential immunoprotection for tissue transplants [103-106].It has been known for many years that Sertoli cells are immuno-privileged cells and do not elicit an autoimmune response [103]. Recent studies have indicated that these cells may confer their immunoprotection when co-transplanted with either allo- or xeno-genic transplants [106].

As a primary cell line, this new tool will allow for the study of cells from a living individual. Therefore, it is possible to obtain cells from multiple individuals for comparison studies. For example, cells could be isolated from individuals with type IV collagen abnormalities (such as hereditary nephropathy) along with those harboring no

abnormalities. The two populations of cells could then be studied to find regulation or expression differences between the two. Also, similar to cultures from biopsies, the individual from which cells are isolated is still alive after the collection of the tissue. Because each male has two testicle which could be removed either together or at two separate times, this could potentially allow for temporal or treatment studies.

Additionally, these primary cultures also have the potential to be converted into a stronger tool through immortalization. The chance to use either the primary or immortalized cell lines in the clinical canine research setting and as a canine model for the human should allow for potentially great steps in these research arenas.

**Materials and methods**

*Testes collection*

Seven mixed breed dogs, all part of a colony housed at Texas A&M University, at 14 weeks of age were anesthetized following Institutional Animal Care and Usage (IACUC) guidelines. While under anesthesia a single testicle was aseptically removed from each dog. The capsule, epididymus, and blood vessels were then removed leaving only the exposed testicle. The testicle was cut into quarters and immediately placed in Dulbecco's modified Eagle's media: nutrient mixture F12 (DMEM : F12 (1:1)) (Gibco, Grand Island, NY, USA) supplemented with 29 ug/ml gentamicin (Gibco).

*Sertoli cell isolation and culture*

Tissue was minced with sterile scissors and scalpel while still in DMEM:F12 containing antibiotic. The minced tissue was then incubated at 37° C and digested with

0.3 wv/mL Liberase Blendzyme 3 (Roche, Indianapolis, IN, USA) plus glass beads to aid in the digestion, while shaking gently. The supernatant was then collected and any undigested cell aggregates were avoided. The collected cell suspension was washed three times, centrifuged for five minutes at 300 RFC and new media supplemented with gentamicin and 20% Fetal Bovine Serum (FBS) (Invitrogen, Carlsbad, CA, USA) was added. Resuspended cells were then filtered through a 100um filter to remove any cell aggregates. Finally, cells were cultured or frozen for later use.

Cells were cultured by seeding 100mm x 15mm culture treated dishes with sub-confluent densities of cell suspensions plus DMEM:F12 supplemented with gentamicin and 20% FBS. Cultures were incubated at 37° C in the presence of 5% $CO_2$ and allowed to adhere overnight. Once cells were adhered, free-floating cells and debris were removed with the media and new media was added. Cultures were then allowed to grow to confluency.

At 70-80% confluency cells were split by removing the media and incubating with PBS for 15 min at 37° C. This was followed by a ten minute incubation in TrypLE Express stable trypsin replacement reagent (Gibco). Cells were split in a 1:2 ratio into a new flask or culture dish and supplemented DMEM:F12 was added.

Frozen stocks were maintained for later use as well. Cells to be frozen were removed from the culture dish as described above with TrypLE Express. Equal volumes of cell suspension in TrypLE Express and freezing media (DMEM:F12, gentamicin, 20% FBS and 15% DMSO) were added to a cryovial and slowly frozen in a Nalgene Cryo 1° C Freezing Container (Nalge Nunc International, Rochester, NY, USA) placed at -80° C overnight. Frozen cryovials were then transferred to liquid nitrogen for long term storage.

When needed, cryovials stored in liquid nitrogen were removed and thawed in a 37° C water bath. The cell suspension was then immediately added to 10mL of supplemented DMEM:F12. The new suspension was centrifuged at 300 RFC for 5 minutes followed by removal of the supernatant. The pellet containing cells was resuspended in fresh supplemented DMEM:F12 and plated. These cultures were then maintained and split as described above.

*Western blot analysis*

Protein was extracted from cells passaged once using the RIPA Lysis Buffer Kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) according to the manufacturer's protocol. Extracted protein was quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) following manufacturer's protocol and either used immediately or stored at -80° C for later use. Loading dye, consisting of 100mM Tris, 25% Glycerol, 2% SDS, 0.01% Bromophenol Blue, and 10% Beta-Mercaptoethanol at pH 6.8, was added to total protein in a 1:2 ratio and boiled for 5 minutes. 76 ug of total protein was loaded onto a precast Ready Gel 4-15% Tris-HCl (BioRad Inc., Hercules, CA, USA). Proteins were separated by size by electrophoresis at 200 volts for 30-40 minutes and transferred to an Immuno-Blot PVDF membrane (0.2um) (BioRad) by electric current of 350 mA for 1 hour at 4° C. Transfer of proteins was confirmed by staining with Ponceau S. PVDF membrane with proteins bound was then blocked for 2 hours rocking gently at room temperature in 5% non-fat milk TBS solution. After blocking, the membrane was incubated in primary antibody at 4°C overnight. The two primary antibodies and their concentrations used were as follows: SOX9 (H-90) rabbit polyclonal antibody (Santa

Cruz Biotechnology, catalog # sc-20095) at a 1:1000 dilution and Cluterin B (C-18) goat polyclonal antibody (Santa Cruz Biotechnology, catalog # sc-6419) at a 1:1000 dilution. Primary antibody was removed and the membrane was washed in 3% milk TBS solution once, 0.025% Tween 20 (BioRad) 3% milk solution twice, and once in TBS alone. All washes were performed at room temperature gently rocking for 10 minutes each. The membrane was then incubated with secondary antibody for one hour at room temperature, while gently rocking. The secondary antibody used to detect SOX9 was a goat anti-rabbit IgG-HRP antibody (Santa Cruz Biotechnology, catalog # sc-2004) at a 1:20,000 dilution, and to detect Clusterin B, donkey anti-goat IgG-HRP antibody (Santa Cruz Biotechnology, catalog # sc-2020) at a 1:40,000 dilution. The secondary antibody was then removed and the membrane was incubated with Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA, USA) as per manufacturer's protocol. Finally an x-ray film was exposed to the membrane for 30 seconds and developed using a Mini-Medical x-ray film processor (AFP Imaging Corporation, Elmsford, NY, USA).

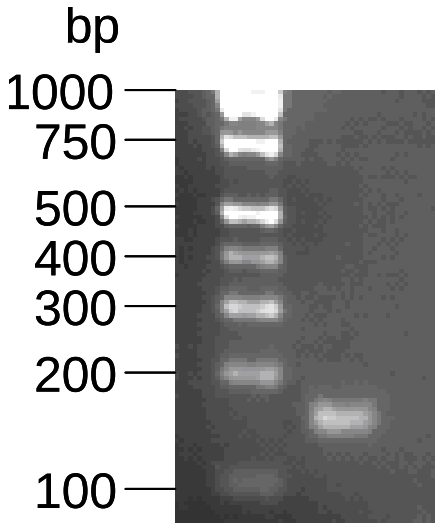


**Figure 9.** Image of SOX9 amplicon (154 base pairs) separated on an agarose gel and stained with ethidium bromide.

*Sequencing cDNA*

Total RNA was isolated using RNA STAT-60 (Iso-Tex Diagnostics, Inc., Friendswood, TX, USA) according to the manufacturer's protocol and cDNA was made from RNA using the Sigma Enhanced Avian HS RT-PCR kit (Sigma Aldrich, St. Louis, MO, USA) following the manufacturer's protocol. PCR was performed to confirm the presence of *SOX9* transcript. Amplification by PCR of cDNA was carried out with each 20ul reaction containing 1.2mM of $MgCl_2$, .25mM of each dNTP, 1.0uM of each primer (forward and reverse), 0.001mg of Bovine Serum Albumin (Promega, Madison, WI), 1 unit of *Taq* DNA Polymerase (Fisher Scientific, Pittsburgh, PA), and 2ul of 1X *Taq* DNA Polymerase Buffer B (Fisher Scientific). Amplification cycling conditions were as follows: 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, then a single cycle at 72°C for 10 minutes. The polymerase, plus half of the polymerase buffer and $MgCl_2$ were added after the first 94°C

cycle. Primers used for *SOX9* were F:CAAGAAAGACCACCCGGATTACAA and R:GGAGGAGGAGTGCGGCGAGT, (product size 154 bases). Products were separated by gel electrophoresis and then visualized using ethidium bromide (figure 9). Products at the correct size were cut out and purified using Qiaex® II Gel Extract Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol. Excised DNA was then used for nucleotide sequencing using the Big Dye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems) and resolved on an ABI 3730 Genetic Analyzer (Applied Biosystems). The resulting sequence was aligned to the known *SOX9* sequence (GenBank accession number AY237827) using Clustal W (http://www.ebi.ac.uk/clustalw/).

# CHAPTER V

# CONCLUSIONS

An important difference between canine models for human diseases and other animal models is the direct benefit the research has on the quality of life for the dog. This was certainly the case for the research done on ARHN in the ECS. For fifty years, HN has devastated the ECS. The autosomal recessive mode of inheritance made identification of HN carriers only possible through their production of dogs affected with the disease. To further complicate the situation, accurate diagnosis of a dog affected with HN requires confirmation of GBM ultrastructural changes and absence of collagen trimers. This can only be done by electron microscopy and immunofluorescence, both of which involve the collection of kidney tissue for analysis (typically through biopsy). Because the diagnosis cannot be made with a simple clinic visit or typical urine and blood tests, it is likely that cases of HN go unreported, meaning the parents of the affected dogs do not get identified as obligate carriers. A second difficulty derived from the diagnosis process is the misdiagnosis of HN when proper diagnostic methods are not performed. One of the characteristics of HN is its course which ultimately leads to end stage renal failure. This characteristic is common to the vast majority of chronic renal diseases, and without thorough examination, such as the diagnostic tests described above, ESRD looks the same no matter the disease causing it. These complications have made identifying carriers of HN difficult for breeders.

The work described in chapter III, in which the mutation causative for HN was characterized, has allowed for the development of a genetic test to identify carriers. This

test will allow breeders to determine if their dogs carry the mutation causing HN, and then to proceed with informed matings. The proper use of this test to judiciously plan matings which will not produce an affected dog, will help to eradicate the disease from the ECS.

In order for the test to be available to breeders in a commercial form, a provisional patent application was filed. This application protected the rights to the test both within the US and world wide one for year after application date. To protect rights to the test beyond the year provided for in the provisional patent, US utility, European, and Australian patents were filed. In order to provide the test commercially, contracts have been established with several companies world wide. US and Canadian tests will be performed by Optigen, of New York, European tests by Antagene, of France, and those from the Asia and extended Pacific regions by Genetic Technologies, of Australia.

While the elucidation of the gene and mutation responsible for ARHN in the ECS described in chapter III and the expression studies on all six type IV collagens described in chapter II were important steps in understanding type IV collagen diseases, expression, and regulation, there is still much more to understand. However, it is possible to build on the research depicted here to make further progress in this research arena. Chapter IV lays out a method for creating a primary Sertoli cell line from testis tissue. Because these cells express all the type IV collagens, they can be used as an excellent model system. This tool, in combination with the dog models of AS, could provide a useful system to study type IV collagens in relation to the disease. Because they are primary in nature, it is possible to collect a single testicle from a dog at one time then the second at a later time, in both affected and unaffected individuals, in order to use the cells for a time course or

treatment study. The strength of this system would also greatly improve if changed to an immortalized cell line. Cells could still originate from affected and unaffected dogs and the immortalization of these cells would build a truly *in vitro* model of AS which could be more easily manipulated than the current primary cell line.

In conclusion, the domestic dog has served as man's companion since its divergence from the wolf, some 15,000 -100,000 years ago [107-109]. The dog's loyalty, reliability, and many diverse physical abilities have made it a staple in our lives. For centuries, we have incorporated the dog into daily routines as companions, hunting partners, rescue helpers, drug and bomb detectors, military sentries, and much more. Humankind's best friend has now been given another important role in relation to us; he now serves as a highly effective research tool to help understand, prevent, and treat hereditary and infectious diseases. The work described here can be added to the ever growing list of successes using the canine model. This work helped lead to a greater understanding of both human and canine forms of AS, and HN, as well as the type IV collagens which are central to these diseases and led to the development of a test that can be used to eliminate the disease from the breed. Finally, using the dog, it is now possible to derive a canine Sertoli cell system to further study AS and the type IV collagens *in vitro*.

## REFERENCES


1. **Online Mendelian Inheritance in Animals, OMIA** [http://omia.angis.org.au/], 2007

2. Ostrander EA, Galibert F, Patterson DF: **Canine genetics comes of age.** *Trends Genet* 2000, **16:**117-124.

3. **American Kennel Club, Complete List of Breeds** [http://www.akc.org/breeds/complete_breed_list.cfm], 2007

4. Neff MW, Rine J: **A fetching model organism.** *Cell* 2006, **124:**229-231.

5. Breen M, Bullerdiek J, Langford CF: **The DAPI banded karyotype of the domestic dog (Canis familiaris) generated using chromosome-specific paint probes.** *Chromosome Res* 1999, **7:**401-406.

6. Kirkness EF, Bafna V, Halpern AL, Levy S, Remington K, Rusch DB, Delcher AL, Pop M, Wang W, Fraser CM, Venter JC: **The dog genome: survey sequencing and comparative analysis.** *Science* 2003, **301:**1898-1903.

7. Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, Clamp M, Chang JL, Kulbokas EJ, 3rd, Zody MC, et al: **Genome sequence, comparative analysis and haplotype structure of the domestic dog.** *Nature* 2005, **438:**803-819.

8. Veit G, Kobbe B, Keene DR, Paulsson M, Koch M, Wagener R: **Collagen XXVIII, a novel von Willebrand factor A domain-containing protein with many imperfections in the collagenous domain.** *J Biol Chem* 2006, **281:**3494-3504.

9. Yoshioka K, Hino S, Takemura T, Maki S, Wieslander J, Takekoshi Y, Makino H, Kagawa M, Sado Y, Kashtan CE: **Type IV collagen alpha 5 chain. Normal distribution and abnormalities in X-linked Alport syndrome revealed by monoclonal antibody.** *Am J Pathol* 1994, **144:**986-996.

10. Kalluri R, Gattone VH, 2nd, Hudson BG: **Identification and localization of type IV collagen chains in the inner ear cochlea.** *Connect Tissue Res* 1998, **37:**143-150.

11. Ninomiya Y, Kagawa M, Iyama K, Naito I, Kishiro Y, Seyer JM, Sugimoto M, Oohashi T, Sado Y: **Differential expression of two basement membrane collagen genes, COL4A6 and COL4A5, demonstrated by immunofluorescence staining using peptide-specific monoclonal antibodies.** *J Cell Biol* 1995, **130:**1219-1229.

12. Kahsai TZ, Enders GC, Gunwar S, Brunmark C, Wieslander J, Kalluri R, Zhou J, Noelken ME, Hudson BG: **Seminiferous tubule basement membrane. Composition and organization of type IV collagen chains, and the linkage of alpha3(IV) and alpha5(IV) chains.** *J Biol Chem* 1997, **272:**17023-17032.

13. Hudson BG, Reeders ST, Tryggvason K: **Type IV collagen: structure, gene organization, and role in human diseases. Molecular basis of Goodpasture and Alport syndromes and diffuse leiomyomatosis.** *J Biol Chem* 1993, **268:**26033-26036.

14. Aumailley M, Gayraud B: **Structure and biological activity of the extracellular matrix.** *J Mol Med* 1998, **76:**253-265.

15. Lowe JK, Guyon R, Cox ML, Mitchell DC, Lonkar AL, Lingaas F, Andre C, Galibert F, Ostrander EA, Murphy KE: **Radiation hybrid mapping of the canine type I and type IV collagen gene subfamilies.** *Funct Integr Genomics* 2003, **3:**112-116.

16. Boyd CD, Weliky K, Toth-Fejel S, Deak SB, Christiano AM, Mackenzie JW, Sandell LJ, Tryggvason K, Magenis E: **The single copy gene coding for human alpha 1 (IV) procollagen is located at the terminal end of the long arm of chromosome 13.** *Hum Genet* 1986, **74:**121-125.

17. Mariyama M, Zheng K, Yang-Feng TL, Reeders ST: **Colocalization of the genes for the alpha 3(IV) and alpha 4(IV) chains of type IV collagen to chromosome 2 bands q35-q37.** *Genomics* 1992, **13:**809-813.

18. Griffin CA, Emanuel BS, Hansen JR, Cavenee WK, Myers JC: **Human collagen genes encoding basement membrane alpha 1 (IV) and alpha 2 (IV) chains map to the distal long arm of chromosome 13.** *Proc Natl Acad Sci USA* 1987, **84:**512-516.

19. Hostikka SL, Eddy RL, Byers MG, Hoyhtya M, Shows TB, Tryggvason K: **Identification of a distinct type IV collagen alpha chain with restricted kidney distribution and assignment of its gene to the locus of X chromosome-linked Alport syndrome.** *Proc Natl Acad Sci USA* 1990, **87:**1606-1610.

20. Oohashi T, Sugimoto M, Mattei MG, Ninomiya Y: **Identification of a new collagen IV chain, alpha 6(IV), by cDNA isolation and assignment of the gene to chromosome Xq22, which is the same locus for COL4A5.** *J Biol Chem* 1994, **269:**7520-7526.

21. Zhou J, Ding M, Zhao Z, Reeders ST: **Complete primary structure of the sixth chain of human basement membrane collagen, alpha 6(IV). Isolation of the**

**cDNAs for alpha 6(IV) and comparison with five other type IV collagen chains.** *J Biol Chem* 1994, **269:**13193-13199.

22. Zhou J, Mochizuki T, Smeets H, Antignac C, Laurila P, de Paepe A, Tryggvason K, Reeders ST: **Deletion of the paired alpha 5(IV) and alpha 6(IV) collagen genes in inherited smooth muscle tumors.** *Science* 1993, **261:**1167-1169.

23. Spiro RG: **Studies on the renal glomerular basement membrane. Nature of the carbohydrate units and their attachment to the peptide portion.** *J Biol Chem* 1967, **242:**1923-1932.

24. Risteli J, Schuppan D, Glanville RW, Timpl R: **Immunochemical distinction between two different chains of type IV collagen.** *Biochem J* 1980, **191:**517-522.

25. Weber S, Engel J, Wiedemann H, Glanville RW, Timpl R: **Subunit structure and assembly of the globular domain of basement-membrane collagen type IV.** *Eur J Biochem* 1984, **139:**401-410.

26. Timpl R, Wiedemann H, van Delden V, Furthmayr H, Kuhn K: **A network model for the organization of type IV collagen molecules in basement membranes.** *Eur J Biochem* 1981, **120:**203-211.

27. Dolz R, Engel J, Kuhn K: **Folding of collagen IV.** *Eur J Biochem* 1988, **178:**357-366.

28. Boutaud A, Borza DB, Bondar O, Gunwar S, Netzer KO, Singh N, Ninomiya Y, Sado Y, Noelken ME, Hudson BG: **Type IV collagen of the glomerular basement membrane. Evidence that the chain specificity of network assembly is encoded by the noncollagenous NC1 domains.** *J Biol Chem* 2000, **275:**30716-30724.

29. Yurchenco PD, Furthmayr H: **Self-assembly of basement membrane collagen.** *Biochemistry* 1984, **23:**1839-1850.

30. Hudson BG, Tryggvason K, Sundaramoorthy M, Neilson EG: **Alport's syndrome, Goodpasture's syndrome, and type IV collagen.** *N Engl J Med* 2003, **348:**2543-2556.

31. Brazel D, Oberbaumer I, Dieringer H, Babel W, Glanville RW, Deutzmann R, Kuhn K: **Completion of the amino acid sequence of the alpha 1 chain of human basement membrane collagen (type IV) reveals 21 non-triplet interruptions located within the collagenous domain.** *Eur J Biochem* 1987, **168:**529-536.

32. Zhou J, Hertz JM, Leinonen A, Tryggvason K: **Complete amino acid sequence of the human alpha 5 (IV) collagen chain and identification of a single-base mutation in exon 23 converting glycine 521 in the collagenous domain to cysteine in an Alport syndrome patient.** *J Biol Chem* 1992, **267:**12475-12481.

33. Timpl R: **Structure and biological activity of basement membrane proteins.** *Eur J Biochem* 1989, **180:**487-502.

34. Gunwar S, Ballester F, Noelken ME, Sado Y, Ninomiya Y, Hudson BG: **Glomerular basement membrane. Identification of a novel disulfide-cross-linked network of alpha3, alpha4, and alpha5 chains of type IV collagen and its implications for the pathogenesis of Alport syndrome.** *J Biol Chem* 1998, **273:**8767-8775.

35. Sado Y, Kagawa M, Naito I, Ueki Y, Seki T, Momota R, Oohashi T, Ninomiya Y: **Organization and expression of basement membrane collagen IV genes and their roles in human disorders.** *J Biochem (Tokyo)* 1998, **123:**767-776.

36. Kleppel MM, Santi PA, Cameron JD, Wieslander J, Michael AF: **Human tissue distribution of novel basement membrane collagen.** *Am J Pathol* 1989, **134:**813-825.

37. Miner JH: **Renal basement membrane components.** *Kidney Int* 1999, **56:**2016-2024.

38. Lees GE, Helman RG, Kashtan CE, Michael AF, Homco LD, Millichamp NJ, Ninomiya Y, Sado Y, Naito I, Kim Y: **A model of autosomal recessive Alport syndrome in English cocker spaniel dogs.** *Kidney Int* 1998, **54:**706-719.

39. Lees GE, Helman RG, Kashtan CE, Michael AF, Homco LD, Millichamp NJ, Camacho ZT, Templeton JW, Ninomiya Y, Sado Y, et al: **New form of X-linked dominant hereditary nephritis in dogs.** *Am J Vet Res* 1999, **60:**373-383.

40. Zheng K, Thorner PS, Marrano P, Baumal R, McInnes RR: **Canine X chromosome-linked hereditary nephritis: a genetic model for human X-linked hereditary nephritis resulting from a single base mutation in the gene encoding the alpha 5 chain of collagen type IV.** *Proc Natl Acad Sci USA* 1994, **91:**3989-3993.

41. Tryggvason K, Martin P: **Alport syndrome and basement membrane collagen, chapter 214.** In *The Metabolic and Molecular Basis of Inherited Disease.* Edited by Shriver C, Beaudet A, Sly W, Valle D, Childs B. Philadelphia: McGraw-Hill; 2001: 5453-5466

42. Kashtan CE: **Alport syndrome and thin glomerular basement membrane disease.** *J Am Soc Nephrol* 1998, **9:**1736-1750.

43. Jais JP, Knebelmann B, Giatras I, De Marchi M, Rizzoni G, Renieri A, Weber M, Gross O, Netzer KO, Flinter F, et al: **X-linked Alport syndrome: natural history in 195 families and genotype- phenotype correlations in males.** *J Am Soc Nephrol* 2000, **11:**649-657.

44. Lemmink HH, Mochizuki T, van den Heuvel LP, Schroder CH, Barrientos A, Monnens LA, van Oost BA, Brunner HG, Reeders ST, Smeets HJ: **Mutations in the type IV collagen alpha 3 (COL4A3) gene in autosomal recessive Alport syndrome.** *Hum Mol Genet* 1994, **3:**1269-1273.

45. Morrison KE, Mariyama M, Yang-Feng TL, Reeders ST: **Sequence and localization of a partial cDNA encoding the human alpha 3 chain of type IV collagen.** *Am J Hum Genet* 1991, **49:**545-554.

46. Longo I, Porcedda P, Mari F, Giachino D, Meloni I, Deplano C, Brusco A, Bosio M, Massella L, Lavoratti G, et al: **COL4A3/COL4A4 mutations: from familial hematuria to autosomal-dominant or recessive Alport syndrome.** *Kidney Int* 2002, **61:**1947-1956.

47. van der Loop FT, Heidet L, Timmer ED, van den Bosch BJ, Leinonen A, Antignac C, Jefferson JA, Maxwell AP, Monnens LA, Schroder CH, Smeets HJ: **Autosomal dominant Alport syndrome caused by a COL4A3 splice site mutation.** *Kidney Int* 2000, **58:**1870-1875.

48. Jefferson JA, Lemmink HH, Hughes AE, Hill CM, Smeets HJ, Doherty CC, Maxwell AP: **Autosomal dominant Alport syndrome linked to the type IV collage alpha 3 and alpha 4 genes (COL4A3 and COL4A4).** *Nephrol Dial Transplant* 1997, **12:**1595-1599.

49. Heidet L, Arrondel C, Forestier L, Cohen-Solal L, Mollet G, Gutierrez B, Stavrou C, Gubler MC, Antignac C: **Structure of the human type IV collagen gene COL4A3 and mutations in autosomal Alport syndrome.** *J Am Soc Nephrol* 2001, **12:**97-106.

50. Harvey SJ, Zheng K, Sado Y, Naito I, Ninomiya Y, Jacobs RM, Hudson BG, Thorner PS: **Role of distinct type IV collagen networks in glomerular development and function.** *Kidney Int* 1998, **54:**1857-1866.

51. Kashtan CE: **Animal models of Alport syndrome.** *Nephrol Dial Transplant* 2002, **17:**1359-1362.

52. Rheault MN, Kren SM, Thielen BK, Mesa HA, Crosson JT, Thomas W, Sado Y, Kashtan CE, Segal Y: **Mouse model of X-linked Alport syndrome.** *J Am Soc Nephrol* 2004, **15:**1466-1474.

53. Cosgrove D, Meehan DT, Grunkemeyer JA, Kornak JM, Sayers R, Hunter WJ, Samuelson GC: **Collagen COL4A3 knockout: a mouse model for autosomal Alport syndrome.** *Genes Dev* 1996, **10:**2981-2992.

54. Miner JH, Sanes JR: **Molecular and functional defects in kidneys of mice lacking collagen alpha 3(IV): implications for Alport syndrome.** *J Cell Biol* 1996, **135:**1403-1413.

55. Lu W, Phillips CL, Killen PD, Hlaing T, Harrison WR, Elder FF, Miner JH, Overbeek PA, Meisler MH: **Insertional mutation of the collagen genes Col4a3 and Col4a4 in a mouse model of Alport syndrome.** *Genomics* 1999, **61:**113-124.

56. Jansen B, Thorner PS, Singh A, Patterson JM, Lumsden JH, Valli VE, Baumal R, Basrur RK: **Animal model of human disease: hereditary nephritis in Samoyed dogs.** *Am J Pathol* 1984, **116:**175-178.

57. Thorner P, Jansen B, Baumal R, Valli VE, Goldberger A: **Samoyed hereditary glomerulopathy. Immunohistochemical staining of basement membranes of kidney for laminin, collagen type IV, fibronectin, and Goodpasture antigen, and correlation with electron microscopy of glomerular capillary basement membranes.** *Lab Invest* 1987, **56:**435-443.

58. Cox ML, Lees GE, Kashtan CE, Murphy KE: **Genetic cause of X-linked Alport syndrome in a family of domestic dogs.** *Mamm Genome* 2003, **14:**396-403.

59. Krook L: **The pathology of renal cortical hypoplasia in the dog.** *Nord Vet-Med* 1957, **9:**161-176.

60. Hood JC, Savige J, Hendtlass A, Kleppel MM, Huxtable CR, Robinson WF: **Bull terrier hereditary nephritis: a model for autosomal dominant Alport syndrome.** *Kidney Int* 1995, **47:**758-765.

61. Hood JC, Huxtable C, Naito I, Smith C, Sinclair R, Savige J: **A novel model of autosomal dominant Alport syndrome in Dalmatian dogs.** *Nephrol Dial Transplant* 2002, **17:**2094-2098.

62. Tryggvason K, Martin, P.: **Alport syndrome and basement membrane collagen,** chapter 214**.** In *The Metabolic and Molecular Basis of Inherited Disease.* Edited by Shriver CR, Beaudet, A.L., Sly, W.S., Valle, D., Childs, B. Philadelphia: McGraw-Hill; 2001: 5453-5466

63. Barker DF, Hostikka SL, Zhou J, Chow LT, Oliphant AR, Gerken SC, Gregory MC, Skolnick MH, Atkin CL, Tryggvason K: **Identification of mutations in the COL4A5 collagen gene in Alport syndrome.** *Science* 1990, **248:**1224-1227.

64. Wiersma AC, Millon LV, Hestand MS, Van Oost BA, Bannasch DL: **Canine COL4A3 and COL4A4: sequencing, mapping and genomic organization.** *DNA Seq* 2005, **16:**241-251.

65. Bernards MA, Valli, V.E.: **Familial renal disease in Samoyed dogs.** *Canadian Veterinary Journal* 1977, **18:**181-189.

66. Mochizuki T, Lemmink HH, Mariyama M, Antignac C, Gubler MC, Pirson Y, Verellen-Dumoulin C, Chan B, Schroder CH, Smeets HJ, et al.: **Identification of mutations in the alpha 3(IV) and alpha 4(IV) collagen genes in autosomal recessive Alport syndrome.** *Nat Genet* 1994, **8:**77-81.

67. Zheng K, Perry J, Harvey SJ, Sado Y, Ninomiya Y, Jefferson B, Jacobs R, Hudson BG, Thorner PS: **Regulation of collagen type IV genes is organ-specific: evidence from a canine model of Alport syndrome.** *Kidney Int* 2005, **68:**2121-2130.

68. Gross O, Netzer KO, Lambrecht R, Seibold S, Weber M: **Meta-analysis of genotype-phenotype correlation in X-linked Alport syndrome: impact on clinical counselling.** *Nephrol Dial Transplant* 2002, **17:**1218-1227.

69. Pfaffl MW: **A new mathematical model for relative quantification in real-time RT-PCR.** *Nucleic Acids Res* 2001, **29:**e45.

70. Efron B: **Bootstrap methods: Another look at the Jackknife.** *Ann Statistics* 1979, **7:**1-26.

71. English PB, Winter H: **Renal cortical hypoplasia in a dog.** *Aust Vet J* 1979, **55:**181-183.

72. Freudiger U: **Die kongenitale nierenrindenhypoplasie beim bunten cocker-spaniel.** *Schweizer Arch Tierheilk* 1965, **107:**547-566.

73. Persson F, Persson S, Asheim A: **Renal cortical hypoplasia in dogs: A clinical study on uraemia and secondary hyperparathyroidism.** *Acta Vet Scand* 1961, **2:**68-84.

74. Koeman JP, Ezilius JW, Biewenga WJ, van den Brom WE, Gruys E: **[Familial nephropathy in cocker spaniels].** *Dtsch Tierarztl Wochenschr* 1989, **96:**174-179.

75. Macdougall DF, Nash AS, Cattanach BM: **Control scheme for familial nephropathy in cocker spaniels.** *Vet Rec* 1987, **121:**134.

76. Robinson WF, Huxtable CR, Gooding JP: **Familial nephropathy in cocker spaniels.** *Aust Vet J* 1985, **62:**109-112.

77. Steward AP, Macdougall DF: **Familial nephropathy in the cocker spaniel.** *J Small Anim Pract* 1984, **25:**15-24.

78. Potter JS, McSporran KD, James MP: **A suspected case of familial nephropathy in the cocker spaniel.** *N Z Vet J* 1985, **33:**65-66.

79. Macdougall D: **Pathogenesis of familial nephropathy in the English cocker spaniel. .** In *Proceedings of the 40th Annual Meeting of the American College of Veterinary Pathologists; Baltimore, MD*. 1989: 27.

80. Lees GE, Wilson PD, Helman RG, Homco LD, Frey MS: **Glomerular ultrastructural findings similar to hereditary nephritis in 4 English cocker spaniels.** *J Vet Intern Med* 1997, **11:**80-85.

81. Lees GE, Helman RG, Homco LD, Millichamp NJ, Hunter JF, Frey MS: **Early diagnosis of familial nephropathy in English cocker spaniels.** *J Am Anim Hosp Assoc* 1998, **34:**189-195.

82. Knebelmann B, Breillat C, Forestier L, Arrondel C, Jacassier D, Giatras I, Drouot L, Deschenes G, Grunfeld JP, Broyer M, et al: **Spectrum of mutations in the COL4A5 collagen gene in X-linked Alport syndrome.** *Am J Hum Genet* 1996, **59:**1221-1232.

83. Ding J, Stitzel J, Berry P, Hawkins E, Kashtan CE: **Autosomal recessive Alport syndrome: mutation in the COL4A3 gene in a woman with Alport syndrome and posttransplant antiglomerular basement membrane nephritis.** *J Am Soc Nephrol* 1995, **5:**1714-1717.

84. Pescucci C, Mari F, Longo I, Vogiatzi P, Caselli R, Scala E, Abaterusso C, Gusmano R, Seri M, Miglietti N, et al: **Autosomal-dominant Alport syndrome: natural history of a disease due to COL4A3 or COL4A4 gene.** *Kidney Int* 2004, **65:**1598-1603.

85. Vega BT, Badenas C, Ars E, Lens X, Mila M, Darnell A, Torra R: **Autosomal recessive Alport's syndrome and benign familial hematuria are collagen type IV diseases.** *Am J Kidney Dis* 2003, **42:**952-959.

86. Baker KE, Parker R: **Nonsense-mediated mRNA decay: terminating erroneous gene expression.** *Curr Opin Cell Biol* 2004, **16:**293-299.

87. Jansen B, Thorner P, Baumal R, Valli V, Maxie MG, Singh A: **Samoyed hereditary glomerulopathy (SHG). Evolution of splitting of glomerular capillary basement membranes.** *Am J Pathol* 1986, **125:**536-545.

88. Jansen B, Valli VE, Thorner P, Baumal R, Lumsden JH: **Samoyed hereditary glomerulopathy: serial, clinical and laboratory (urine, serum biochemistry and hematology) studies.** *Can J Vet Res* 1987, **51:**387-393.

89. Yurchenco PD, Smirnov S, Mathus T: **Analysis of basement membrane self-assembly and cellular interactions with native and recombinant glycoproteins.** *Methods Cell Biol* 2002, **69:**111-144.

90. Miner JH, Sanes JR: **Collagen IV alpha 3, alpha 4, and alpha 5 chains in rodent basal laminae: sequence, distribution, association with laminins, and developmental switches.** *J Cell Biol* 1994, **127:**879-891.

91. Sund M, Maeshima Y, Kalluri R: **Bifunctional promoter of type IV collagen COL4A5 and COL4A6 genes regulates the expression of alpha5 and alpha6 chains in a distinct cell-specific fashion.** *Biochem J* 2005, **387:**755-761.

92. Greer KA, Higgins MA, Cox ML, Ryan TP, Berridge BR, Kashtan CE, Lees GE, Murphy KE: **Gene expression analysis in a canine model of X-linked Alport syndrome.** *Mamm Genome* 2006, **17:**976-990.

93. Pollner R, Schmidt C, Fischer G, Kuhn K, Poschl E: **Cooperative and competitive interactions of regulatory elements are involved in the control of divergent transcription of human Col4A1 and Col4A2 genes.** *FEBS Lett* 1997, **405:**31-36.

94. Heidet L, Borza DB, Jouin M, Sich M, Mattei MG, Sado Y, Hudson BG, Hastie N, Antignac C, Gubler MC: **A human-mouse chimera of the alpha3alpha4alpha5(IV) collagen protomer rescues the renal phenotype in Col4a3-/- Alport mice.** *Am J Pathol* 2003, **163:**1633-1644.

95. Segal Y, Zhuang L, Rondeau E, Sraer JD, Zhou J: **Regulation of the paired type IV collagen genes COL4A5 and COL4A6. Role of the proximal promoter region.** *J Biol Chem* 2001, **276:**11791-11797.

96. Harvey SJ, Perry J, Zheng K, Chen D, Sado Y, Jefferson B, Ninomiya Y, Jacobs R, Hudson BG, Thorner PS: **Sequential expression of type IV collagen networks: testis as a model and relevance to spermatogenesis.** *Am J Pathol* 2006, **168:**1587-1597.

97. Magnol JP, Pin D, Palazzi X, Lacour JP, Gache Y, Meneguzzi G: **[Characterization of a canine model of dystrophic bullous epidermolysis (DBE). Development of a gene therapy protocol].** *Bull Acad Natl Med* 2005, **189:**107-119; discussion 119-121.

98. Merhi RA, Guillaud L, Delouis C, Cotinot C: **Establishment and characterization of immortalized ovine Sertoli cell lines.** *In Vitro Cell Dev Biol Anim* 2001, **37:**581-588.

99. Majumdar SS, Winters SJ, Plant TM: **Procedures for the isolation and culture of Sertoli cells from the testes of infant, juvenile, and adult rhesus monkeys (Macaca mulatta).** *Biol Reprod* 1998, **58:**633-640.

100. Roberts KP, Banerjee PP, Tindall JW, Zirkin BR: **Immortalization and characterization of a Sertoli cell line from the adult rat.** *Biol Reprod* 1995, **53:**1446-1453.

101. Tabuchi Y, Ohta S, Yanai N, Obinata M, Kondo T, Fuse H, Asano S: **Development of the conditionally immortalized testicular Sertoli cell line TTE3 expressing Sertoli cell specific genes from mice transgenic for temperature sensitive simian virus 40 large T antigen gene.** *J Urol* 2002, **167:**1538-1545.

102. Walther N, Jansen M, Ergun S, Kascheike B, Tillmann G, Ivell R: **Sertoli cell-specific gene expression in conditionally immortalized cell lines.** *Adv Exp Med Biol* 1997, **424:**139-142.

103. Barker CF, Billingham RE: **Immunologically privileged sites.** *Adv Immunol* 1977, **25:**1-54.

104. Whitmore WF, Gittes RF: **Studies on the prostate and testis as immunologically privileged sites.** *Cancer Treat Rep* 1977, **61:**217-222.

105. Shamekh R, El-Badri NS, Saporta S, Pascual C, Sanberg PR, Cameron DF: **Sertoli cells induce systemic donor-specific tolerance in xenogenic transplantation model.** *Cell Transplant* 2006, **15:**45-53.

106. Emerich DF, Hemendinger R, Halberstadt CR: **The testicular-derived Sertoli cell: cellular immunoscience to enable transplantation.** *Cell Transplant* 2003, **12:**335-349.

107. Vila C, Savolainen P, Maldonado JE, Amorim IR, Rice JE, Honeycutt RL, Crandall KA, Lundeberg J, Wayne RK: **Multiple and ancient origins of the domestic dog.** *Science* 1997, **276:**1687-1689.

108. Savolainen P, Zhang YP, Luo J, Lundeberg J, Leitner T: **Genetic evidence for an East Asian origin of domestic dogs.** *Science* 2002, **298:**1610-1613.

109. Sablin MV, Khlopachev GA: **The earliest Ice Age dogs: evidence from Eliseevichi 1.** *Current Anthropology* 2002, **43:**795–799

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