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SEQUENCE ANALYSIS OF THE ANGIOTENSIN II TYPE 1 RECEPTOR (AGTR1) GENE FOR MUTATIONS CONTRIBUTING TO PULMONARY HYPERTENSION IN THE CHICKEN (*GALLUS GALLUS)*

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Abstract

Our multidisciplinary group at the University of Arkansas has been investigating the suitability of the chicken as a medical model for pulmonary arterial hypertension (PAH) in humans. There are several forms of PAH in humans arising from elevated pulmonary arterial pressure and pulmonary vascular resistance. Spontaneous cases of PAH are known as idiopathic PAH (IPAH), where the exact physiological causes are not known. IPAH patients that do not respond to standard treatments have a prognosis of only a few years. Currently, there is no acceptable animal model for IPAH. As part of our effort to pursue the chicken as model for IPAH, we have mapped chromosomal regions associated with susceptibility to ascites, an industry term for PAH in chickens. One region identified contains the angiotensin II type 1 receptor gene (AGTR1), a gene known to be associated with particular forms of PAH in humans. My project was to sequence the exonic regions of AGTR1 from chickens selected for susceptibility and resistance to ascites. I identified a total of 15 single nucleotide polymorphisms (SNPs) in intronic regions and 9 affecting exonic sequences that distinguish resistant and susceptible birds. One of the SNPs alters the encoded protein, but more sequencing data is needed to confirm the presence of this SNP. The 24 SNPs have become the basis for further genotypic efforts using quantitative polymerase chain reaction high-resolution melt (qPCR-HRM) assays to determine the association of alternative alleles of AGTR1 with PAH in the chicken. This knowledge will help the poultry industry in genetic selection to reduce incidence of ascites. Characterization of the genetic determinants of ascites in the chicken will also identify specific gene networks and further our understanding of PAH in humans.

Introduction

Pulmonary arterial hypertension (PAH) is currently accepted as being classified into three main types: 1) idiopathic pulmonary arterial hypertension (IPAH), 2) familial pulmonary arterial hypertension (FPAH), and 3) pulmonary arterial hypertension related to associated conditions or risk factors (APAH) (Simmonneau et al., 2004). IPAH and FPAH have also been classified as types of primary pulmonary hypertension, meaning they have no known cause. APAH has been classified as secondary pulmonary hypertension because the hypertension is believed to be due to an underlying cause.

IPAH results in increases in pulmonary arterial pressure and in pulmonary vascular resistance, which ultimately causes right ventricular failure and death if untreated or unresponsive to treatment (Widlitz and Barst, 2003). The increased vascular resistance in patients with pulmonary hypertension is due to vasoconstriction, thrombosis in situ, and vascular remodeling (Rubin, 1997). Primary pulmonary hypertension, including IPAH, is defined as having a mean pulmonary artery pressure of 25 mmHg or greater at rest or 30 mmHg or greater during exercise, with no related underlying condition (Widlitz and Barst, 2003). IPAH is diagnosed in one to two new patients per million each year, with an average survival of 2.5 years after initial diagnosis (Rubin, 1997).

Adults with primary pulmonary hypertension generally develop plexiform lesions and seemingly irreversible pulmonary vascular changes (Widlitz & Barst, 2003). These vascular changes are phenotypic changes in the endothelial and smooth muscle cells in pulmonary arteries and are influenced by cell proliferation, apoptosis, matrix proteins, and matrix turnover. Upregulation of the transcription factor Oct-4 has been shown to be involved in the irregular vasculature of the pulmonary arterial smooth muscle cells in IPAH (Firth et al., 2010). Hemodynamic influences are also important in the etiology of PAH. Additionally, gene expression in pulmonary vascular cells is related to environmental factors, receptors, growth factors, signaling pathways, and genetic influences, all of which can interact with each other in the disease process.

Serotonin metabolism has been shown to be central to the etiology of PAH (MacLean, 2007). Serotonin (5-hydroxytryptamine, 5HT) can be synthesized in the pulmonary endothelium and released to either interact with serotonin cell surface receptors or enter the smooth muscle cells via the serotonin transporter (SERT). Serotonin can act to induce proliferation and contraction of pulmonary smooth muscle cells through 5HT receptors on the vascular epithelium. The actions of 5HT receptors have been shown to be involved in PAH in humans (Humbert et al., 2004).

The Renin-Angiotensin Aldosterone System (RAAS) is a hormonal cascade that is important in the pathogenesis of cardiovascular disorders (Atlas, 2007). The RAAS functions in maintaining the homeostasis of arterial pressure, tissue perfusion, and extracellular volume. The cascade begins with the secretion of renin by the juxtaglomerular cells of the kidneys. In the extracellular space, renin functions to cleave ten amino acids from the N-terminus of angiotensinogen, which is constitutively

secreted by the liver. The cleaved angiotensinogen is an inactive ten amino acid peptide known as angiotensin I. Two amino acids from the C-terminus of angiotensin I are cleaved by membrane-bound angiotensin-converting enzyme (ACE), creating the octapeptide, angiotensin II. Angiotensin II is the primary biologically active product of the RAAS and acts as a potent vasoconstrictor.

At least four types of angiotensin receptors have been identified, but the type 1 receptor (angiotensin II type 1 receptor, or AGTR1) is involved in most of the known physiology of angiotensin II (Atlas, 2007). Cardiovascular effects of angiotensin II and AGTR1 include vasoconstriction, increased blood pressure, increased cardiac contractility, and vascular and cardiac hypertrophy. Angiotensin II and AGTR1 have also been shown to stimulate aldosterone synthesis by the adrenal cortex, inhibit renin release, increase sodium reabsorption in the kidneys, and affect the sympathetic nervous system. Angiotensin II and AGTR1 also mediate effects on cell growth and proliferation, inflammatory responses, and oxidative stress. AGTR1 contains the seven transmembrane domains characteristic of the G protein-coupled receptor superfamily and is found on many cell types in angiotensin II target organs.

The RAAS has been shown to be genetically involved in human PAH (Chung et al., 2009). A specific polymorphism in the angiotensin-converting enzyme (ACE) gene has been shown to be associated with primary pulmonary hypertension (IPAH) and right ventricular function (Abraham et al., 2003). Increased expression of ACE has also been seen in the plexiform lesions of PAH (Orte et al., 2000). Recent research has shown that certain variants of AGTR1 are significantly related to PAH. Specifically, an adenine (A)/cytosine (C) substitution at position 1166 in the 3' untranslated region of AGTR1 is significantly associated with early onset IPAH in humans (Chung et al., 2009). This SNP occurs within a potential target site for the miRNA-155, a microRNA (miRNA) binding site (Ceolotto et al., 2011). They theorized that miRNA-155 can target the 1166A allele, but not the 1166C allele. A study of hypertensive subjects found that AGTR1 protein expression was negatively correlated with miRNA-155 expression and that AGTR1 protein expression was significantly greater in subjects homozygous for the C allele than in heterozygous (CA) or homozygous A subjects. This would be consistent with miRNA-155 repression of the 1166A alleles and lack of repression of the 1166C SNP. Overexpression of AGTR1 protein would then lead to higher blood pressure.

Ascites, the industry term for PAH in chickens, results from a fast growth rate and causes significant mortality in chickens (Pavlidis et al., 2007). Ascites is among the most common and most deadly cardiovascular conditions in modern lines of broiler chickens (Olkowski, 2007). Increased genetic selection for fast growth rate and the creation of diets that stimulate fast growth contribute to the development of ascites in the broiler chicken (Julian, 1993). Pulmonary hypertension in poultry causes right ventricular hypertrophy, valvular insufficiency, increased venous pressure, and ascites. Because the chicken heart has a thin-walled right ventricle and a muscular right atrioventricular valve, pulmonary hypertension causes heart failure and death quickly in

the chicken. Also, the broiler chicken is relatively small, but has very large breasts, causing increased pressure on the air sacs of the relatively small lungs.

The etiology of ascites in the broiler chicken begins with the high basal metabolic rate required for fast growth (Government of Alberta, 2008). Broilers are susceptible to ascites when their pulmonary vascular capacity cannot tolerate the cardiac output necessary for meeting the metabolic requirements of rapid growth, which could be due to anatomical or functional restrictions (Wideman et al., 2007). This inadequate capacity causes a deficit in oxygen, which in turn causes the right ventricle to increase the amount of blood pumped to the lungs to be oxygenated (Government of Alberta, 2008). Because the right ventricle is thin in the chicken, it often expands due to the increase in blood flow. The increased blood flow causes increased pulmonary arterial pressure and right ventricular hypertrophy. More red blood cells are produced by the chicken to increase the oxygen carrying capacity of the blood, but more red blood cells also cause the blood to thicken and further increase vascular resistance. Valvular insufficiency causes right ventricular failure and the associated backflow causes liver edema, which leads to leakage of plasma into the body cavity. This leads to an accumulation of fluid in the abdomen (ascites). In the poultry industry the term is also "water belly" and leads to either mortality or condemnation at the plant. Ascites can be treated in the chicken with beta agonists and dietary arginine, both of which increase ventilation and pulmonary blood flow (Currie, 1999). Also, feed restriction is used to cause a tachycardia that may protect birds from the brachycardia associated with hypoxia-induced ascites.

There are several currently used animal models for human pulmonary hypertension, with the most common being derived from exposing the animal to chronic hypoxia or to monocrotaline injury (Stenmark et al., 2009). Animals used include rats, mice, piglets, calves, and macaques. Fawn-hooded rats are commonly used because pulmonary hypertension manifests most severely in these animals. However, none of these develops the plexogenic arteriopathies found in humans and chickens. Our collaborative group at the University of Arkansas is examining whether ascites in the chicken (*Gallus gallus*) is a medical model for PAH/IPAH. Ascites is easily induced in the chicken and lines of PAH resistant and susceptible chickens have been developed. There are genetic and pathophysiological similarities between ascites in the chicken and PAH in the human. Chickens with ascites form plexogenic arteriopathies that are indistinguishable from plexiform lesions found in the lungs of humans with advanced PAH (Wideman et al., 2011). Genetic evidence for the chicken being an appropriate medical model for PAH can be found in our group's identification of chromosomal regions associated with ascites in the chicken that contain genes that have been found to be involved in PAH in humans (Chung et al., 2009; MacLean, 2007; Orte et al., 2000; Smith, 2009).

A number of methods have been employed to induce PAH in the chicken, including injection of bacterial lipopolysaccharide (LPS), injection of micro-particles of cellulose, and exposure to hypoxia to simulate high altitudes, such as growth in a hypobaric chamber (Chapman et al., 2005; Currie, 1999; Pavlidis, 2007).

Dr. Nicholas Anthony (University of Arkansas) has developed divergently selected lines of broiler chickens that are resistant (RES) or susceptible (SUS) to ascites using a hypobaric chamber to induce the disease (Pavlidis, 2007). The chamber simulated high altitude conditions via a partial vacuum to lower the partial pressure of oxygen in the chamber. The RES and SUS lines were created based on sib-selection over successive generations in the hypobaric chamber.

Our group then used a cross of the RES and SUS lines to map several chromosomal segments contributing to PAH in the chicken (Smith, 2009). One of those regions contains a serotonin receptor-transporter (5HT receptor), which has been associated with some forms of human PAH (MacLean, 2007). Another region contained the ACE gene. Those genes are under investigation by other students in the laboratory. A third region contains the gene AGTR1, which is located on chromosome 9 and codes for the angiotensin II type 1 receptor. As already mentioned, this gene and the RAAS have been shown to be involved in PAH in humans (Atlas, 2007; Chung et al., 2009). The implications of our preliminary data is that mis-regulation of angiotensin II production by ACE and the subsequent response by AGTR1 may be a contributing factor for development of ascites.

Understanding the genetics of PAH in the chicken model will identify specific gene networks and will advance understanding of the underlying processes leading to PAH in humans. This knowledge may also lead to genetic selection in the poultry industry to lessen the economic losses associated with ascites.

The region containing the AGTR1 gene on chromosome 9 (Gga9) was previously associated with ascites in the chicken through a whole genome association study and then confirmed using variable number tandem repeat (VNTR) genotyping (Smith, 2009). Further research in our laboratory has associated this region with ascites in our lines and in three commercial lines (Sriram Krishnamoorthy, personal communication). Therefore, this region appears to contain a major determinant of ascites susceptibility in chickens. A region in the human genome that contains the AGTR1 gene has been associated with some forms of PAH, thus it is likely that mutations affecting the AGTR1 gene could contribute to ascites in chickens.

My research project was to sequence the AGTR1 gene from the resistant and susceptible lines to determine whether mutations could be found that represent alternative alleles of AGTR1. I then developed qPCR-HRM assays for the alternative alleles of AGTR1 for use in genotyping a larger collection of DNAs. Additional genotype data will determine whether these particular mutations

are associated with susceptibility to PAH. As depicted in Figure 1, the chicken AGTR1 gene's three exons are distributed across 20,979 bases (Gga 9: 13,475,625 – 13,496,603), with the entire coding sequence located in exon 3 (International Chicken Genome Sequencing Consortium, 2004). The three exons produce a final mRNA transcript of 1427 bases. The first two exons are non-coding while the final exon encodes a predicted 359 amino acid protein that is the receptor.

Materials and Methods

DNA Isolation

DNAs were previously isolated by Sriram Krishnamooorthy, Ph.D. candidate University of Arkansas, from chickens that were then phenotyped in a hypobaric chamber. These DNAs have been previously validated by others in our laboratory for PCR amplification of numerous regions.

Primer Design for Polymerase Chain Reaction (PCR)

The genomic region representing AGTR1 was downloaded from the UCSC genome browser. Primer3 (http://frodo.wi.mit.edu/primer3/) was used to design oligonucleotide primers covering all of the exonic regions. Primers were synthesized by Eurofins-Operon. PCR conditions were then optimized for primer pairs.

Polymerase Chain Reaction (PCR)

PCR mixtures were assembled incorporating H_2O, PCR Buffer, deoxyribonucleotide triphosphate (dNTPs), Taq polymerase, primers, and target DNA. Twenty µl PCR mixtures contained 1X PCR Buffer (50 mM TrisCl, 1 mM $MgCl_2$, 3 mg/ml BSA), 0.2 mM dNTPs, 3 units Taq polymerase, 2 µl target DNA, and 1 µM forward and reverse primer mixture. Reactions for sequencing consisted of 200 µl of the same concentrations of 1X PCR Buffer, 0.2 mM dNTPs, 1 µM forward and reverse primer mixture, but differed by using 10 units Taq polymerase, and 5 µl target DNA. Reactions were cycled in one of four PCR machines in our laboratory to obtain amplicons of the three exons of AGTR1 in the resistant and susceptible birds.

Gel Electrophoresis for Quality of PCR Product

A portion of the PCR product was resolved on a 1.5% agarose gel to evaluate the quality of the product. Five µl of PCR product was mixed with 5 µl of loading dye prior to loading into gel. The loading dye used was 10 µg/ml ethidium bromide (EthBr), 15% Ficoll, 20mM EDTA, 0.1% Bromophenol Blue 0.1% Xylene Cyanol. Molecular weight ladder was pGEM5 digested with Sau3A. Gels were submerged in 0.5X TBE with 0.5 µg/ml EthBr and electrophoresed for approximately 5-10 minutes at 40 Volts
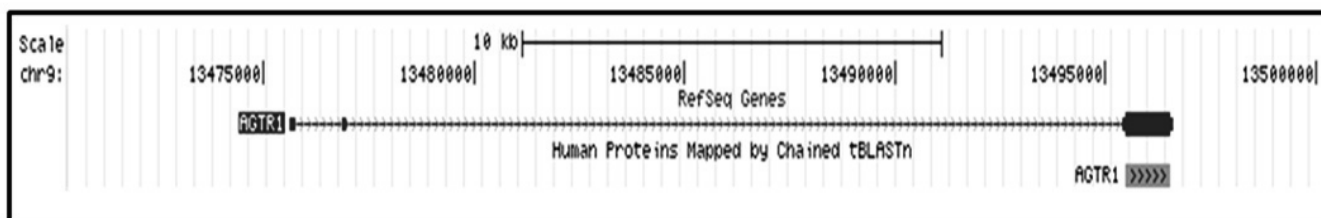

Figure 1: UCSC genome browser view of the chicken AGTR1 gene on chromosome 9 (International Chicken Genome Sequencing Consortium, 2004). http://genome.ucsc.edu.

(V) and then for approximately 90 minutes at 70 V.

Gel Imaging

Gels were imaged either using an Ultraviolet Transilluminator captured with a CCD camera and FlashPoint FPG software, or the gels were scanned at 600nm using a Model 9600 Typhoon Imager (GE HealthCare) and analyzed with ImageQuant Software.

Purification and Quantification of PCR product

To purify DNA samples for sequencing, reactions were diluted 1x with TE (10 mM TrisCl, 1 mM EDTA, pH 7.5) and extracted with phenol:chloroform:isoamyl alcohol (50:48:2) and then with chloroform:isoamyl alcohol (24:1). The solution was brought to 0.3 M with sodium acetate and DNA precipitated by addition of 3 volumes of 95% ethanol. The mixture was incubated at -20°C for 1-2 hours and then DNAs were collected by centrifugation (10 min at 10,000 rpm). The pellet was rinsed with cold 70% ethanol, dried under vacuum and redissolved in 10 mM TrisCl, 0.1 mM EDTA. DNA was quantified using a Model TKO 100 Hoefer Scientific Instruments DNA Fluorometer according to the manufacturer's instructions.

DNA Sequencing and Analysis

Purified PCR products (5-20 ng) were mixed with 3.4 pM of individual primers, and submitted to the UA DNA Resource Center for capillary sequencing. The sequence files were aligned using Seqman software from DNAStar.

Quantitative PCR – High Resolution Melt (qPCR-HRM) Assays for SNPlotyping

Primers were designed that flank single nucleotide polymorphisms (SNPs) to generate a short amplicon (generally less than 60 base pairs). The oligonucleotide primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) and were synthesized by Eurofins-Operon.

Twenty µl PCR reactions each contained 1X PCR Buffer (50 mM TrisCl, 1 mM MgCl$_2$, 3 mg/ml BSA), 0.2 mM dNTPs, 3 units Taq polymerase, 1 µl target DNA, 1 µM forward and reverse primer mixture, H$_2$O, and an intercalating dye (SYBR Green, LCGreen, or EvaGreen). The qPCR-HRM assay was performed on a BioRad CFX96 Real-Time System. The protocol used included the following steps: 1) 95°C for 30 seconds, 2) 95°C for 15 seconds, 3) 50°C for 15 seconds, 4) 72°C for 30 seconds and plate read, 5) Repeat steps 2-4 39 times, 6) 72°C for 3 minutes, 7) 95°C for 3 minutes, 8) 65°C for 3 minutes, and 9) Gradient from 65°C-90°C at 0.1°C increments for 10 seconds with plate read at each increment.

Results

I designed four pairs of oligonucleotide primers that amplify the three exonic regions of the AGTR1 gene. The size of exon 3 required the use of two pairs of primers, while exons 1 and 2 required one pair each. Two additional pairs of primers were designed to flank exon 3 because the original pair flanking one portion of exon 3 produced no PCR product. All primers were optimized for annealing temperatures and cycling conditions using chicken genomic DNA. The primer pair names, sequences, and optimized PCR conditions are shown in Tables 1 and 2.

The primer pair for exon 1 (AGTR1 X1 F1/R1) was optimized for 20 µl PCRs but no product was obtained when a 200 µl PCR was performed for sequencing. The AGTR1 X3-2 F1/R1 primer pair did not produce a PCR product.

Quality amplicons of exons 2 and 3 were obtained from 200 µl PCR reactions, purified, and sequenced. Sequences were obtained from two of the three AGTR1 exonic regions from several resistant and susceptible birds (usually 3 of each). The sequences were aligned with the AGTR1 gene from the 2006 genome assembly of the Red Jungle Fowl (*Gallus gallus*) sequence. The alignments for exon 2 and exon 3 are presented in Figures 2 and 3, respectively.

Comparisons between Jungle Fowl, resistant, and susceptible sequences identified 24 single nucleotide polymorphisms (SNPs), four of which affected the AGTR1 coding sequence. All identified SNPs are listed in Table 3. Fourteen SNPs were found in the intronic sequence 5' of exon 2 and three SNPs were found within exon 2. One SNP was found in the intronic sequence 5' of exon 3. Six SNPs were identified in exon 3 including one SNP in the 3' untranslated region (UTR).

Five pairs of primers were designed to amplify specific SNPs in exons 2 and 3 for high resolution melt analyses. Two primers designed to amplify regions containing SNPs were optimized using genomic chicken DNA and are ready to be used in a SNPlotyping application. Three of the five primer pairs designed to amplify the SNP regions were not optimized.

A LCGreen qPCR-HRM assay to SNPlotype multiple resistant and susceptible chickens was completed for the SNP located at base 2513. The AGTR1_2513F/R primer pair was used and the melt curve is shown in Figure 4.

Discussion

Several polymorphisms were identified that establish that there are at least two forms of the AGTR1 gene in our RES and SUS lines (Pavlidis, 2007). The 14 SNPs found in the intronic region 5' of exon 2 and the three SNPs in exon 2 do not affect the coding region for the AGTR1 protein, as these SNPs are in non-coding regions. Two SNPs, one in the intronic region 5' of exon 3 and one in exon 3, need more sequence from additional chickens for confirmation. Sequence data was only obtained from two individuals, both resistant, for each of these two SNPs. The intronic SNP would have no effect on the protein sequence, but the SNP in exon 3 is in the first position of a proline codon at position 82 of the protein (Pro82) and would change the encoded amino acid to serine. Proline residues are normally considered as disrupters of secondary structure. This proline is located within the extracellular portion of the alpha helical transmembrane II domain (G Protein-Coupled Receptor Database, 2011). Because of its location outside of the hydrophobic membrane, replacement of the proline with a hydrophilic serine would not alter the hydrophilicity of the region but certainly could alter a secondary structure. Pro82 is highly conserved in human, cow, mouse, rat, opossum, lizard, frog, and zebrafish (See Figure 5). Four other SNPs found in exon 3 are located in the third codon position (i.e., the wobble position) and would be silent with respect to the encoded protein. The specific codon changes are shown in Table 3.

Table 1: Primer information. Sequence in capital letters indicate exonic sequences, with lowercase for intronic sequences. Numbers in SNP primer pair names correspond to the base pair location of the SNP in the mRNA for exonic SNPs and relative to the transcription start site in the gene for intronic SNPs.

Amplified Region	Primer Pair Name	Fragment Length (bp)	Forward Sequence (5' - 3')	Reverse Sequence (5' - 3')
Exon 1	AGTR1 X1 F1/R1	808	gtcacggcacactcccttagcaa	ggtccttcctgcgcaacttca
Exon 2	AGTR1 X2 F1/R1	844	tcaaaaatcaccgaaggcagtg	caacaagcaaaagggactgacg
Exon 3-1	AGTR1 X3-1 F1/R1	785	gcagattaattgccgactattcca	AAAGCCACAGACCGTCATGTTC
Exon 3-2	AGTR1 X3-2 F1/R1	760	TCACGAATCCGACGTACCATGT	gatgtctggactgcagatgga
Exon 3-1	AGTR1 X3-1 F2/R2	836	gtgcccacaatccagttttc	CCACAGACCGTCATGTTCAA
Exon 3-2	AGTR1 X3-2 F2/R2	697	CTTGCTGGTGTGGCCAGT	ctgggactgcagatggattt
Intron Indel/SNP	AGTR1_2228_2235 F/R	50	gtattcttccaagcaaatcc	agtgcttgtccataccatga
Exon 2 SNP	AGTR1_2513 F/R	50	aaaaggacttttgtctatttgga	AGTCCCCTCTTCTCCCTGCTC
Exon 3 SNP	AGTR1_21618 F/R	50	GCCGTCATCATTCATCG	TCATGTTCAAGTTCTCTGCAAAA
Exon 3 SNP	AGTR1_21945 F/R	52	CATGTAATAACAGACTGCAAAAT	AGGGCATAGCTGTATCCACAA
Exon 3 SNP	AGTR1_22050 F/R	59	TTTTTATGTTTTCTTTGGAA	TGTATTTTATTAGCTGAAGGAAG

Table 2: Optimized PCR conditions for primer pairs.

| Primer Name	Step 1: Initial Denaturation	Step 2: Denaturation	Step 3: Annealing	Step 4: Extension	Number of Cycles (repeat steps 2-4)	Step 5: Final Extension
AGTR1 X1 F1/R1	90°C, 60s	90°C, 20s	55°C, 20s	72°C, 2min	40	72°C, 3min
AGTR1 X2 F1/R1	90°C, 60s	90°C, 20s	53°C, 20s	72°C, 2min	40	72°C, 3min
AGTR1 X3-1 F1/R1	90°C, 60s	90°C, 20s	50°C, 20s	72°C, 2min	40	72°C, 3min
AGTR1 X3-2 F1/R1	90°C, 60s	90°C, 20s	50°C, 20s	72°C, 2min	40	72°C, 3min
AGTR1 X3-1 F2/R2	Not Optimized					
AGTR1 X3-2 F2/R2	Not Optimized					
AGTR1_2228_2235 F/R	90°C, 30s	90°C, 15s	50°C, 15s	72°C, 30s	44	72°C, 3min
AGTR1_2513 F/R	90°C, 30s	90°C, 15s	50°C, 15s	72°C, 30s	44	72°C, 3min
AGTR1_21618 F/R	Not Optimized					
AGTR1_21945 F/R	Not Optimized					
AGTR1_22050 F/R	Not Optimized					
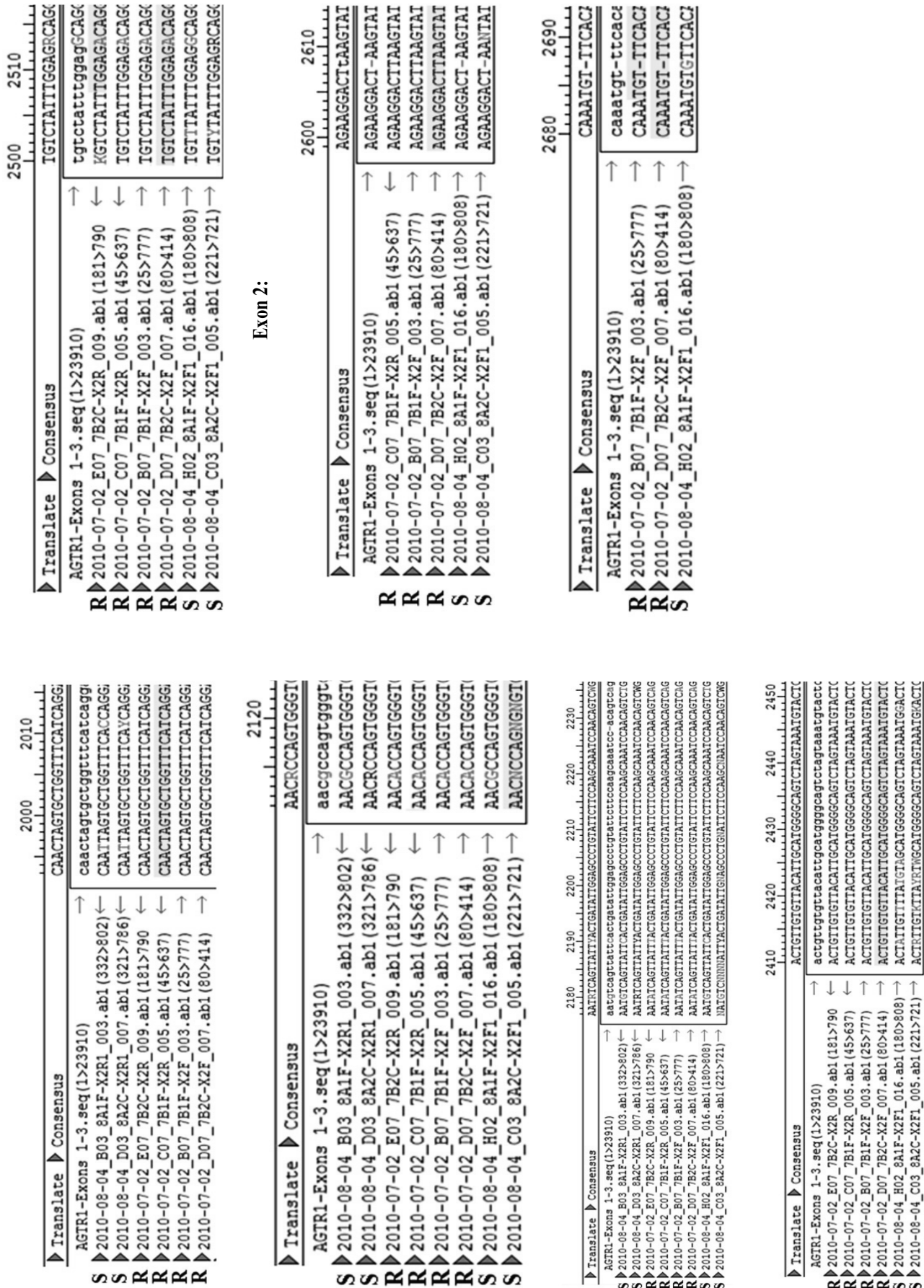
Figure 2: AGTR1 SNPs in the intronic region 5' of exon 2 and in exon 2. **(R)** denotes PAH-resistant birds and **(S)** denotes PAH-susceptible birds. Sequences are aligned with the 2006 Red Jungle Fowl sequence assembly (International Chicken Genome Sequencing Consortium, 2004).

Figure 3: AGTR1 SNPs in the intronic region 5' of exon 3, in exon 3, and in the UTR 3' of exon 3. (**R**) denotes PAH-resistant birds and (**S**) denotes PAH-susceptible birds. Sequences are aligned with the 2006 Red Jungle Fowl sequence assembly (International Chicken Genome Sequencing Consortium, 2004). Sequence within the coding region of exon 3 is shown with the associated protein sequence (bottom reading frame).

Table 3: SNP Information. Jungle Fowl genotype was obtained from the 2006 assembly of Red Jungle Fowl (*Gallus gallus*) (International Chicken Genome Sequencing Consortium, 2004). Codon Change is relative to the Jungle Fowl sequence. *More sequence data is needed to confirm.

SNP Region	Locus (bp)	Jungle Fowl Genotype	Resistant Genotype(s)	Susceptible Genotype(s)	Codon Change
Intronic region 5' of exon 2	1996	CC	CC	TT	N/A
Intronic region 5' of exon 2	2011	TT	TT	CC, C/T	N/A
Intronic region 5' of exon 2	2114	GG	AA	GG, G/A	N/A
Intronic region 5' of exon 2	2180	GG	AA	GG, G/A	N/A
Intronic region 5' of exon 2	2190	CC	TT	CC, C/T	N/A
Intronic region 5' of exon 2	2228	No Base	AA	AA	N/A
Intronic region 5' of exon 2	2235	AA	AA	TT, A/T	N/A
Intronic region 5' of exon 2	2413	GG	GG	AA, A/G	N/A
Intronic region 5' of exon 2	2418	GG	GG	TT, G/T	N/A
Intronic region 5' of exon 2	2422	CC	CC	TT, C/T	N/A
Intronic region 5' of exon 2	2423	AA	AA	GG, G/A	N/A
Intronic region 5' of exon 2	2425	TT	TT	AA, A/T	N/A
Intronic region 5' of exon 2	2448	TT	TT	GG, G/T	N/A
Intronic region 5' of exon 2	2503	CC	CC	TT, C/T	N/A
First base of exon 2	2513	GG	AA	GG, G/A	N/A
Exon 2	2608	No Base	TT	No Base	N/A
Exon 2	2687	No Base	No Base	GG	N/A
*Intronic region 5' of exon 3	20991	AA	TT	No Data	N/A
*Exon 3	21361	CC	C/T	No Data	CCA (Pro) to TCA (Ser)
Exon 3	21618	TT	TT	CC, C/T	CGT (Arg) to CGC (Arg)
Exon 3	21945	CC	CC	TT, C/T	ATC (Ile) to ATT (Ile)
Exon 3	21987	CC	TT	CC, C/T	ATT (Ile) to ATC (Ile)
Exon 3	22050	AA	GG	AA, A/G	AAA (Lys) to AAG (Lys)
UTR 3' of exon 3	22210	TT	CC	TT, C/T	N/A

The SNP found in the 3' UTR of exon 3 was not found to be analogous to the 3' UTR SNP found to be associated with PAH by Chung et al (2009). The SNP in the human AGTR1 gene was 86 bases 3' to the terminator codon, but the SNP found in the chicken was 13 bases 3' to the stop codon. However, this SNP may still be of importance in terms of its potential effects on miRNA binding sites. There are several miRNA binding sites located in the 3' UTRs of the human AGTR1 mRNA, so SNPs located in one of these binding sites could have an effect on miRNA function and gene expression (Elton et al., 2010). Perhaps the SNP found in the 3' UTR of the chicken is localized in a miRNA binding site or creates a novel miRNA binding site, both of which could affect miRNA function and gene expression.

New primers may need to be designed to amplify exon 3 and three of the SNP regions, while a different strategy or primer redesign may be necessary to obtain sequence for exon 1. Sequence data needs to be obtained for exon 1 and additional sequence data is needed for the first portion of exon 3. Also, more work can be done to determine whether any particular AGTR1 allele is associated with PAH susceptibility. One approach used in our laboratory is the development of qPCR-HRM (quantitative PCR High-Resolution Melt) assays for SNPlotyping a collection of DNAs representing different susceptible and resistant lines. Two of the five SNP primers I designed are ready for this analysis, whereas three others need further optimization or need to be redesigned. Statistical analysis of the frequency of each SNPlotype can be performed to determine whether there is an association of any of these SNPs with ascites phenotype. In addition, based on frequencies and concordance, it can be determined which SNPs are in linkage and define different particular haplotypes (alleles) for AGTR1.

The melt curve obtained from a qPCR-HRM assay for the SNP located at base 2513 showed insufficient allelic discrimination (data not shown). That is, the melting temperatures were not significantly different between the resistant and susceptible chickens so as to determine that there are or are not two alleles. Additional analysis using qPCR-HRM assays are being pursued to examine additional SNPs identified in AGTR1.

A Ph.D. candidate in the lab is currently using qPCR on cDNA to study expression levels of AGTR1 in different tissues from resistant and susceptible chickens. He has detected significant differences in expression of AGTR1 in RNA from whole blood. With qPCR-HRM assays we should be able to correlate expression levels with particular alleles to determine whether the allelic effect is associated with differences in expression or the protein product.

Further genetic analysis of the different forms of the AGTR1 gene from both susceptible and resistant birds will lead to insight into how particular mutations in the gene may contribute to the development of ascites in the chicken. It is this knowledge of the genetics of ascites in chickens that will lead to genetic selection to reduce ascites in chickens, and further develop the chicken as a medical model for PAH in humans. Availability of an animal medical model will provide the means to develop and evaluate new treatments for PAH in humans.
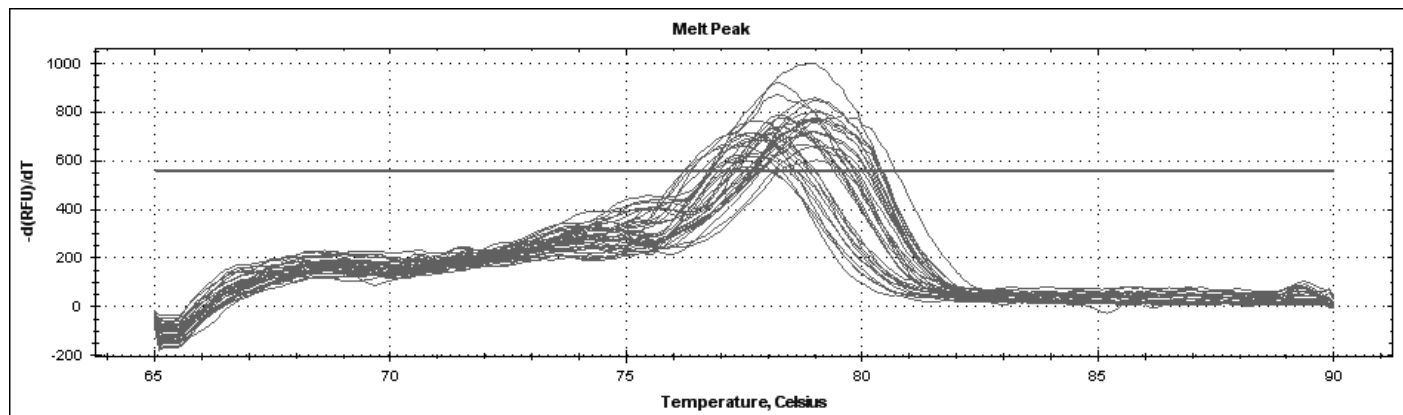
Figure 4. Melt Curve for SNP at base 2513. The peaks represent melting temperatures of each sample. There is insufficient discrimination between RES and SUS chickens to determine if two alleles are or are not present.

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Human        FLLNLALADLCFLLTLPLWAVYTAMEYRWPFGNYLCKIASASVSFNLYAS
Chimpanzee   FLLNLALADLCFLLTLPLWAVYTAMEYRWPFGNYLCKIASASVSFNLYAS
Cow          FLLNLALADLCFLLTLPLWAVYTAMEYRWPFGNYLCKIASASVSFNLYAS
Mouse        FLLNLALADLCFLLTLPLWAVYTAMEYRWPFGNHLCKIASASVSFNLYAS
Rat          FLLNLALADLCFLLTLPLWAVYTAMEYRWPFGNHLCKIASASVSFNLYAS
Opossum      FLLNLALADLCFLMTLPLWAAYTAMEYRWPFGNCLCKIASAGISFNLYAS
Lizard       FLFNLALADLCFIVTLPLWAATTAMEYRWPFGNCLCKLTSAAASFNLYAS
Frog         FLMNLALSDLCFVITLPLWAVYTAMHYHWPFGDLLCKIASTAITLNLYTT
Zebrafish    YIGNLALADLTFVITLPLWAVYTALGYHWPFGVALCKISSYVVLVNMYAS
G.gallus     FLLNLALADLCFLITL**P**LWAAYTAMEYQWPFGNCLCKLASAGISFNLYAS
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Figure 5. Alignment of AGTR1 proteins from selected vertebrates. The region of AGTR1 from residue 66 to 115 (based on *G. gallus*) are shown for the indicated organisms. Protein sequences were extracted from GenBank and aligned at http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi. The proline residue affected by the SNP is underlined and bold (see text).

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Mentor Comments: Dr. Rhoads' comments place Russell's work into the larger context of a major interdisciplinary research initiative that showcases some of the most exciting work taking place on the University of Arkansas campus.

Russell Burks' sequence analyses of the AGTR1 gene is part of a collaborative project between several research groups on the University of Arkansas campus investigating Pulmonary Arteriole Hypertension (PAH) in the chicken. PAH results in a disease known as Ascites in the poultry industry with annual costs estimated at over $100,000,000. During the past 15 years, Dr. Nicholas Anthony in Poultry Science developed ascites susceptible and ascites resistant lines of chickens. We then collaborated on mapping the gene regions contributing to ascites phenotype. One gene region contained the AGTR1 gene which, in humans, has been implicated in some forms of PAH. Sriram Krishnamoorthy (Ph.D. student in Dr. Anthony's group) has confirmed that the region of AGTR1 is associated with Ascites phenotype in the experimental lines and in commercial lines.

Therefore, Russell took on the task of sequencing the AGTR1 gene from the resistant and susceptible lines. He used PCR to amplify the exonic regions from multiple chickens. Sequences of those regions revealed a number of single nucleotide polymorphisms (SNPs). Some of these SNPs are not in exons, while a few affect exonic sequences including one that would alter the AGTR1 protein. Although none of these SNPs may be the actual mutation that contributes to ascites susceptibility/resistance, they are excellent markers for investigating this region further. AGTR1 is part of the angiontensin pathway which regulates vasoconstriction and kidney function. Dr. Robert Wideman (Poultry Science) and Dr. Heidi Kluess (Health Science, Kinesiology, Recreation and Dance) have been investigating the pharmacology of vasoconstriction in lung arterioles of the chicken. Adnan Al-Rubaye (Ph.D. student in my laboratory) is investigating the expression levels of AGTR1 in different tissues from resistant and susceptible chickens. Therefore, Russell's contribution is a set of molecular tools that will allow us to correlate particular AGTR1 alleles with gene expression levels, physiological responses, and prediction of ascites phenotype.

What is truly exciting about this is that the research at the University of Arkansas has been demonstrating that the chicken may very well be one of the best medical models for PAH in humans. The AGTR1 work is part of that and demonstrates that the chicken has many of the same underlying physiological and genetic bases for PAH as found

in humans. Thus, we are not only going to understand the disease to minimize the impact on the poultry industry, but may also develop an excellent model for understanding and developing new treatments for the disease in humans.