CHARACTERIZATION OF A MAJOR LOCUS AFFECTING COAT

COLOR REDDENING IN CATTLE

An Undergraduate Research Scholars Thesis

by

TASHA WOMACK

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Clare Gill

May 2015

Major: Animal Science

TABLE OF CONTENTS

ABSTRACT

Characterization of a major locus affecting coat color reddening in cattle. (May 2015)

Tasha Womack
Department of Animal Science
Texas A&M University

Research Advisor: Dr. Clare Gill
Department of Animal Science

In cattle, differences in base coat color are attributed to the enzymatic activity specified by the melanocortin-1 receptor *(MC1R)* locus with alleles coding for black (E^D), red (*e*), and wild-type (E^+). These alleles have a presumed dominance model of $E^D > E^+ > e$. In Nellore-Angus F_2 cattle, some $E^D E^+$ heterozygotes have displayed various degrees of red pigmentation when predicted to have a black background. This variation has been associated with a major locus on *Bos taurus* chromosome (BTA) 6 interacting with *MC1R*. The objective of this study is to identify the causative mutation on BTA6 and characterize the effect of the mutation on expression of genes in the melanocyte pigmentation pathway.

This region of BTA6 coincides with a cluster of *tyrosine kinase receptor* genes including *PDGFRA*, *KIT*, and *KDR*. We hypothesize that a structural variant in or near *PDGFRA*, *KIT* or *KDR* causes this novel reddening phenotype in cattle. First, the critical interval containing the reddening locus was refined to BTA6:70,714,167-71,404,818 using imputed and phased HD SNP data in our F_2 Nellore-Angus mapping population. Then SNP and indels from whole-genome sequencing of the founders of the mapping population were used to impute the region to sequence scale to find variants concordant with recessive inheritance of the reddening alleles

from Nellore. Next, skin biopsies from biological triplicates were collected for each genotypic combination of the extension locus ($E^D E^D$, $E^D E^+$, $E^+ E^+$) and the reddening locus (NN, NA, AA). A TRI Reagent isolation method and RNeasy mini kit was used to extract total RNA (RIN > 6.5). Primers for realtime RT-qPCR were designed for each of the tyrosine kinase receptor genes (*PDGFRA*, *KIT* or *KDR*), *MC1R* and *ASIP*.

The relative expression of each gene was calculated and no significant differences in expression of the candidate genes (*PDGFRA*, *KIT*, and *KDR*) categorized by genotype at MC1R and reddening were found; however, long-range regulatory effects on *PDGFRA*, *KIT* or *KDR* still cannot be ruled out. Among individuals genotyped as heterozygous EDE+ at the extension locus, those that were homozygous Nellore at reddening exhibited significantly ($P < 0.05$) lower levels of expression for *MC1R* than those that were homozygous Angus at reddening. Reexamination of HD SNP data identified *CHIC2* as another positional candidate.

ACKNOWLEDGEMENTS

CHAPTER I

INTRODUCTION

In many breeds of domesticated livestock, coat color became one of the traits put under intense artificial selection and, ultimately, color became part of the identity of many breeds. Hulsman et al. [1] explains that availability of either premiums or discounts due to coat color phenotypes can have a direct impact on producers' breeding strategies so having an understanding of the genes and mechanisms involved in coat color pigmentation of cattle is desirable. In cattle, differences in base color are attributed to mutations in the melanocortin-1 receptor (*MC1R*) gene, also termed the extension locus (reviewed by Hulsman et al. [1]), with alleles coding for black (E^D), red (*e*), and wild-type (E^+). These alleles are thought to follow a dominance model, in which $E^D > E^+ > e$.

Extension and *Agouti* loci

Previous studies have shown the *MC1R* locus has a major function in the regulation and synthesis of pheomelanin (red to yellow pigment) versus eumelanin (black to brown pigment) in the melanogenesis pathway. Eumelanin synthesis is induced by binding of α-melanocyte-stimulating hormone (α-MSH) to the melanocortin-1 receptor, whereas pheomelanin synthesis occurs when agouti signaling protein (ASP) blocks the receptor.

In *Bos taurus* cattle, five alleles of *MC1R* on bovine chromosome 18 have been found: three versions of the E^+ allele produce a functional receptor and interact with alleles of the *Agouti* (*A*) locus to produce cattle that are reddish brown with varying amounts of black [2,4]; the dominant

4

allele (E^D) is a point mutation (c.296C > T), resulting in an amino acid change from leucine to proline, which creates a constitutively active receptor that results in eumelanin production and cattle with black hides [2]; the recessive allele (*e*) is due to a frame shift mutation (c.311delG) that produces a prematurely terminated, non-functional receptor causing the sole production of the pheomelanin pigment resulting in cattle with red hides [2,3]. Olson [5] reports that there is complete dominance of E^D over E^+ and *e* and that when an E^D allele is present an active receptor is produced regardless of the activity of the *A* locus. A resultant increase in enzyme activity of *MC1R* leads to eumelanin production and the dominant black coat color.

The *Agouti* (*ASIP*) locus, a complex locus that has 17 alleles in mice, is also a coat color determinant. Wild-type agouti signaling protein (ASP) produced in the hair follicle inhibits *MC1R* by blocking the receptor and preventing access by α-MSH, which causes pheomelanin production in hair follicle melanocytes [7]. Variation at the *A* locus on chromosome 13 in cattle remains incompletely understood, but similar to the situation in mice, the agouti-signaling protein (ASP) is a known antagonist of *MC1R* that inhibits α-melanocyte-stimulating hormone (α-MSH) induced eumelanin synthesis by blocking the receptor.

Major locus found on BTA6 interacting with *MC1R*

In a previous study by the Animal Genomics Laboratory in the Department of Animal Science at Texas A&M [1], a major locus was found that interacts with *MC1R* causing a reddening effect in cattle that were expected to be black. Hulsman et al. [1] compared resultant genotypes of a F_2 population of ½ *Bos indicus* (Nellore) ½ *Bos taurus* (Angus) cattle with photographs and discovered that $E^D E^D$ homozygotes were black and $E^+ E^+$ homozygotes ranged in color from

yellow to black as expected. However, $E^D E^+$ heterozygotes often exhibited various degrees of

reddening and were not black as predicted by the dominance series [1]. A major locus that

affects reddening was detected between 60 and 73 Mb on BTA6 (Btau4.0 build of the bovine

genome sequence) and at 72 Mb by single-marker association and Bayesian methods [1]. The

posterior mean of the genetic variance for this region accounted for 43.75% of the genetic

variation in reddening [1]. This region coincides with a cluster of *tyrosine kinase receptor* genes

including *PDGFRA*, *KIT*, and *KDR*; *KIT* has previously been associated with coat color spotting

in a number of species. These genes became strong candidate genes for the reddening phenotype

because tyrosinase is the rate limiting enzyme in the synthesis of both pheomelanin and

eumelanin [8]. Hulsman et al. [1] concluded that one of these three coding genes, or an

associated structural or regulatory variant, is probably responsible for the majority of the

variation in the reddening phenotype. Furthermore, an interval between 71,708,424 and

72,478,577 bp on BTA6 was constructed [1], which eliminates the coding sequences of *KIT* and

KDR as candidates. The regulatory effects of these genes cannot be ruled out, but the location of

PDGFRA still falls within the refined interval allowing it to be the strongest positional candidate

for the reddening phenotype.

PDGFRA is a member of the platelet derived growth factors (PDGF) family that consists of two

receptors (α and β) and four ligands (A-D). A direct link to the role of *PDGFRA* in determination

of cattle coat color has not been previously documented, although its function and cellular

responses have been extensively studied in the developing mouse. In respect to pigmentation,

PDGFRA has been shown to have roles in gastrulation signaling [9] and in the development of

neural-crest derived pigment cells, including proliferation, migration, and tissue remodeling [10].

6

A genome-wide association study in a Chinese Holstein population implicated *PDGFRA* and *KIT* in coat color by identifying significant (P<0.05) SNPs near and within these genes on BTA6 associated with proportion of black and teat color [11]. *KIT* is known to produce mutations resulting in white coat color or white spotting in numerous species including cattle [12]. Furthermore, during melanogenesis, *KIT* has been identified to drive melanocyte migration from the neural crest along the dorsolateral pathway [1].

The objectives of this study are to (1) identify the causative mutation on BTA6 for the reddening phenotype and (2) to characterize the effect of the mutation on expression of genes in the melanocyte pigmentation pathway. We hypothesize that a structural variant in or near *PDGFRA*, *KIT* or *KDR* causes this novel reddening phenotype in cattle.

CHAPTER II

METHODS

All work involving animals has been approved by the Institutional Animal Care and Use Committee (IACUC); AUP 2011-291.

The same Nellore-Angus population as used in [1] was used for this study. Briefly, the population consists of large full-sibling families produced by multiple ovulation and embryo transfer. The founders of the population (7 Nellore bulls and 6 Angus cows) have been sequenced and single nucleotide polymorphisms (SNP) were discovered by alignment with the Hereford UMD3.1 reference sequence; the founders and the F_1 generation have been genotyped with a high density (770,000) SNP chip; the F_2 generation was genotyped with a 50,000 SNP chip. The whole population has now been imputed to sequence scale, meaning that the missing information from the F_2 generation has been filled in with information from the previous two generations.

For objective 1, the interval containing the reddening locus was refined to identify the causative mutation. First, the grandparent's sequence alignments spanning the critical interval on BTA6 were reviewed to confirm whether there was any indication of large structural variants (e.g. deletions, insertions or inversions) in the region. Next, a catalog of SNP and any biallelic structural variants was created. Then these data were phased with fastPHASE 1.4.0 [13] and breed of origin of each haplotype was determined by tracing the inheritance of Nellore or Angus alleles through the three-generation pedigree. The data from individuals that were recombinant

in the region were manually examined to identify variants concordant with the reddening phenotype.

For objective 2, biological triplicates were collected for each genotypic combination of the extension locus ($E^D E^D$, $E^D E^+$, $E^+ E^+$) and the reddening locus (NN, NA, AA) (Table 1). Skin biopsies were obtained using a clean 'V' cut ear notcher that produced half inch wide ear pieces, which were immediately covered with 4M guanidinium isothiocyanate (GITC). Skin was removed from each side of the ear notch, transferred in duplicate to 1 mL GITC, frozen on dry ice and stored at -80°C. A TRI Reagent isolation method from Brannan et al. [14] was used to ensure the recovery of total RNA that was high in both quantity and integrity. Briefly, a frozen ear notch from each animal was ground to powder using a stainless steel Biopulverizer that had been previously cooled with liquid nitrogen. The pulverized biopsies (n = 21) were then transferred into 2.2 mL TRI Reagent and forcefully mixed by use of an 18 gauge needle and 3 mL syringe. Total RNA in the aqueous phase was collected after centrifugation and separated with 200 μl of 1-bromo-3-chloropropane (BCP), followed by a second separation with a combination of 200 μl BCP and 400 μl TRI Reagent, and a final separation using 200 μl of BCP alone. The aqueous phase from the BCP separation was added to an equal volume of 70% ethanol. Each sample was then applied to an RNeasy Mini spin column. Total RNA from each column was eluted once with 50 μl of nuclease-free water and then eluted again using the original 50 μl eluate and stored at -80˚C. The isolated total RNA was quantified and assessed for quality using Nanodrop® spectrophotometry and an Agilent® 2100 Bioanalyzer, respectively. The Bioanalyzer processed the Eukaryote Total RNA Nano Series II chip using capillary electrophoresis and Agilent® 2100 Bioanalyzer Expert software. Electropherogram plots and

Table 1 Selection categories for skin biopsies. Biological triplicates were collected for each genotypic combination of the extension locus and the reddening locus; *samples for homozygote individuals at both the extension locus and reddening locus were collected as controls.

Extension locus genotype	Reddening locus genotype		
$E^D E^D$	AA* (n = 3)		
	NN* (n = 3)		
$E^D E^+$	AA (n = 3)		
	AN (n = 3)		
	NN (n = 3)		
$E^+ E^+$	AA* (n = 3)		
	NN* (n = 3)		

10

RNA integrity numbers (RINs) computed by the Expert software were used to evaluate the RNA isolated for each sample. With adequate RNA quantity and quality (RIN > 6.5), the elutions were converted to cDNA using a SuperScript® VILO^{TM} cDNA synthesis kit. Briefly, each reaction tube contained 4 µl 5X VILO^{TM} Reaction Mix, 2 µl 10X SuperScript® Enzyme Mix, 1.0 µg of total RNA and enough nuclease-free water to bring the reaction volume to 20 µl. The tube contents were gently mixed and incubated at 25˚C for ten minutes, then 42˚C for 60 minutes and terminated at 85˚C for five minutes. The newly synthesized cDNA was diluted 10-fold with yeast tRNA (25ng/µl) to obtain a concentration of 100 ng/µl of RNA needed for the qRT-PCR reactions.

Primers for realtime qRT-PCR were designed for each of the tyrosine kinase receptor genes (*PDGFRA*, *KIT* or *KDR)*, *MC1R* and *ASIP* (Table 2). These primers, with the exception of the intron-less *MC1R* gene, purposely span an intron to allow for exclusive amplification of cDNA. Housekeeping primers of GAPDH and 18S ribosomal RNA were also selected as internal control genes to normalize the PCRs for the amount of RNA added to the reverse transcription reactions. All primers were run on separate plates and at the same temperature (60˚C) and conditions. Each sample was run in triplicate following the Conventional and Real-time PCR protocol developed in the Bovine Functional Genomics & Proteomics Lab by Dr. Penny Riggs. Briefly, a commercial master mix was used including the reagent *Power* SYBR® by Applied Biosystems, forward and reverse primers, and water. The 96-well plates were set up with 2µl of cDNA template to achieve a 20µl reaction volume and run with "absolute" cycling parameters on an Applied Biosystems 7900HT Fast Real-time PCR System. Relative expression levels of the genes were determined following Livak and Schmittgen (2001). The analysis used the $2^{-\Delta\Delta C_T}$

Table 2 Primer name, location, sequences of forward and reverse primers, and product sizes for both genomic DNA and cDNA.

Primer name	Chromosome	Primer sequences	Product sizes (bp)	
			genomic DNA	cDNA
GAPDH	BTA5	5'-TTC AAC GGC ACA GTC AAG G -3' 5'-ACA TAC TCA GCA CCA GCA TCA C -3'	237	119
18S ribosomal RNA	BTA25	5'-AGA AAC GGC TAC CAC ATC CA -3' 5'-CAC CAG ACT TGC CCT CCA -3'	169	169
PDGFRA	BTA6	5'- ACG TGT CCA AAG GCA GTA CC -3' 5'- GGG AAA AGA TCT CCC AGA GC -3'	NA	126
KIT	BTA6	5'- GTC CAG GAA CTG AGC AGA GG -3' 5'- CCC ATT GTG TTT GAA TGT GC -3'	1818	134
KDR	BTA6	5'- TGA GAG CCC CTG ATT ACA CC -3' 5'- AAA CGT GGG TCT CTG ATT GG -3'	496	86
MC1R	BTA18	5'- GCA GTC CCT TGA CAA AGA GG -3' 5'- CCT GGA GAG TCC TGA GAT TCC -3'	96	96
ASIP	BTA13	5'- TAC CTT GCT GGT CTG CCT GT -3' 5'- CTT TTC CGC TTC ATT TCT GC -3'	1496	181
CHIC2	BTA6	5'- GTC TCG GAG TCT CAG GAT GG -3' 5'- TCA GTC CAA ATA CGG TGA CG -3'	NA	148

Reference: UMD 3.1.1 bovine assembly

method. Cycle threshold values for each reaction were normalized to the geometric mean of the control genes. The average relative expression of the samples that were homozygous dominant at extension and homozygous Angus at reddening individuals were chosen as the calibrator. The raw C_T values of the triplicate samples were averaged before the $2^{-\Delta\Delta C_T}$ values were calculated. Differences in expression levels in cases and controls were established using pairwise *t*-tests.

CHAPTER III

RESULTS

Through interval analysis, a critical interval of BTA6: 71,708,424 – 72,478,577 using Btau 4.0 assembly coordinates was previously established based on recombinant individuals and the assumption that reddening is associated with the Nellore allele and acts as a recessive [1]. For the current study, the F_2 Nellore-Angus population was then imputed and phased using HD SNP data and the two original recombinant individuals that defined the region were able to further refine the interval to BTA6: 70,714,167 – 71,404,818 (Figure 1) now based on UMD3.1 coordinates. The UMD3.1 assembly was chosen over the Btau 4.0 assembly because in the latter assembly there is a large deletion in this region that is an assembly artifact, based on comparison of both assemblies to the bovine optical map (D. Schwartz, personal communication). The coding sequence of *PDGFRA* remained in this newly refined interval.

Although *PDGFRA* remains the strongest candidate, long-range regulatory effects on *KIT* or *KDR* still cannot be ruled out, so we performed qPCR on skin biopsies to uncover differences in expression levels of the genes of interest. The relative expression of each gene (*PDGFRA*, *KIT*, *KDR*, *MC1R*, and *ASIP*), normalized to the geometric mean of GAPDH and 18S and relative to the calibrator is displayed in Figure 2. We observed the most consistent expression among the triplicates for *MC1R*. Among individuals genotyped as heterozygous $E^D E^+$ at the extension locus, those that were homozygous Nellore at reddening exhibited significantly ($P < 0.05$) lower levels of expression for *MC1R* than those that were homozygous Angus at reddening. Although there were no other significant differences detected for *ASIP*, the means for the homozygous

wild-type group were lower than the homozygous dominant group means. This is concordant

with the understanding that wild-types will generally have lower expression levels of *MC1R*

because sometimes the receptor will be activated or blocked depending on the interaction of the

wild-type allele with α-MSH or alleles at the Agouti locus, respectively. Within genotypic

category, relative expression levels of Agouti varied the greatest and we observed no significant

differences in expression. The particularly high variability in expression for the heterozygotes

suggests that the mode of action of the reddening locus on *MC1R* is independent of *ASIP*. When
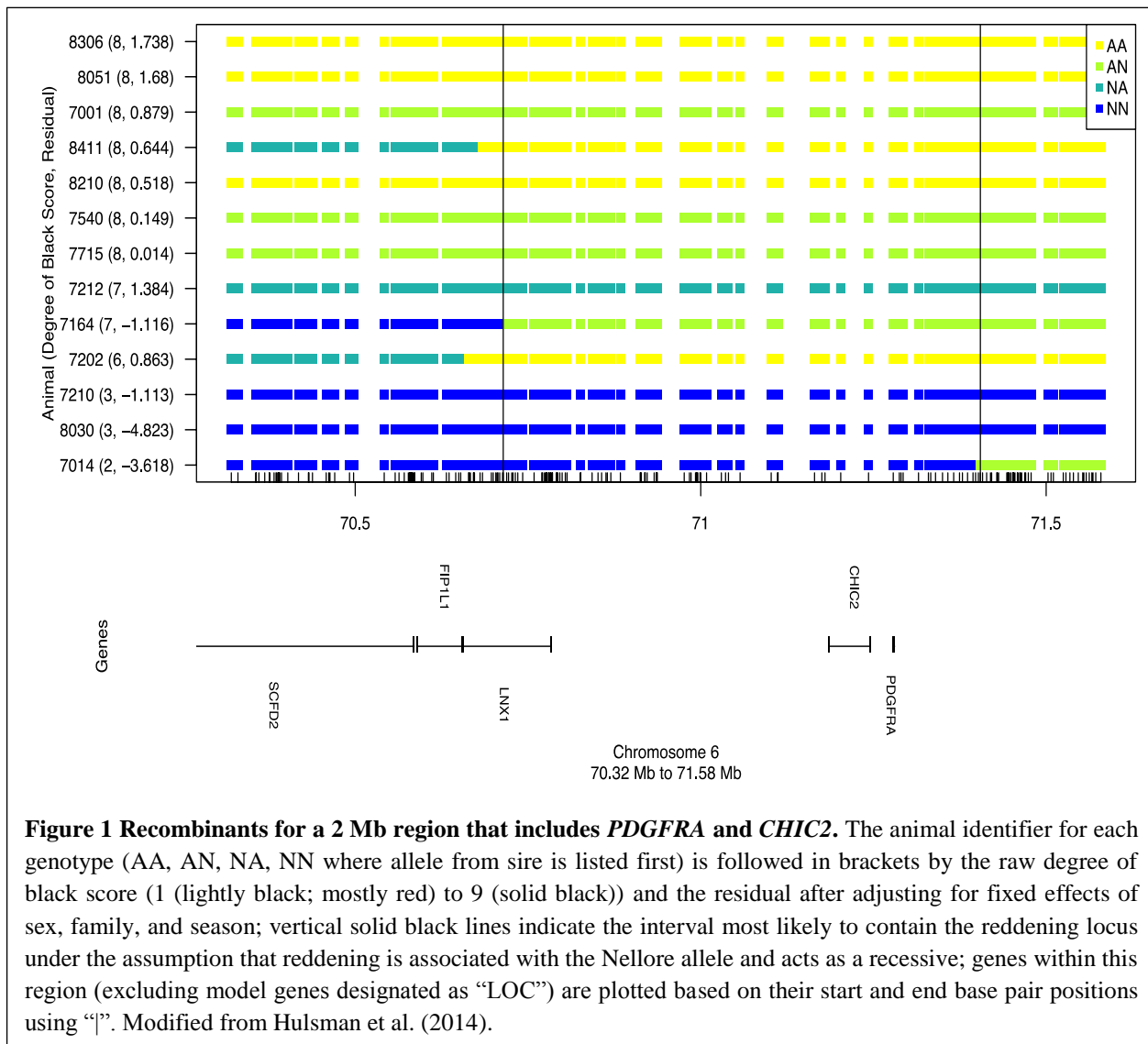
Figure 1 Recombinants for a 2 Mb region that includes *PDGFRA* and *CHIC2*. The animal identifier for each genotype (AA, AN, NA, NN where allele from sire is listed first) is followed in brackets by the raw degree of black score (1 (lightly black; mostly red) to 9 (solid black)) and the residual after adjusting for fixed effects of sex, family, and season; vertical solid black lines indicate the interval most likely to contain the reddening locus under the assumption that reddening is associated with the Nellore allele and acts as a recessive; genes within this region (excluding model genes designated as "LOC") are plotted based on their start and end base pair positions using "|". Modified from Hulsman et al. (2014).

relative expression levels of *ASIP* and *MC1R* are compared between the homozygous for

extension groups, higher levels of Agouti are observed in the homozygous Nellore individuals

Figure 2 Clustered column graphs depicting relative gene expression levels in target genes. Samples were categorized along the x-axis first b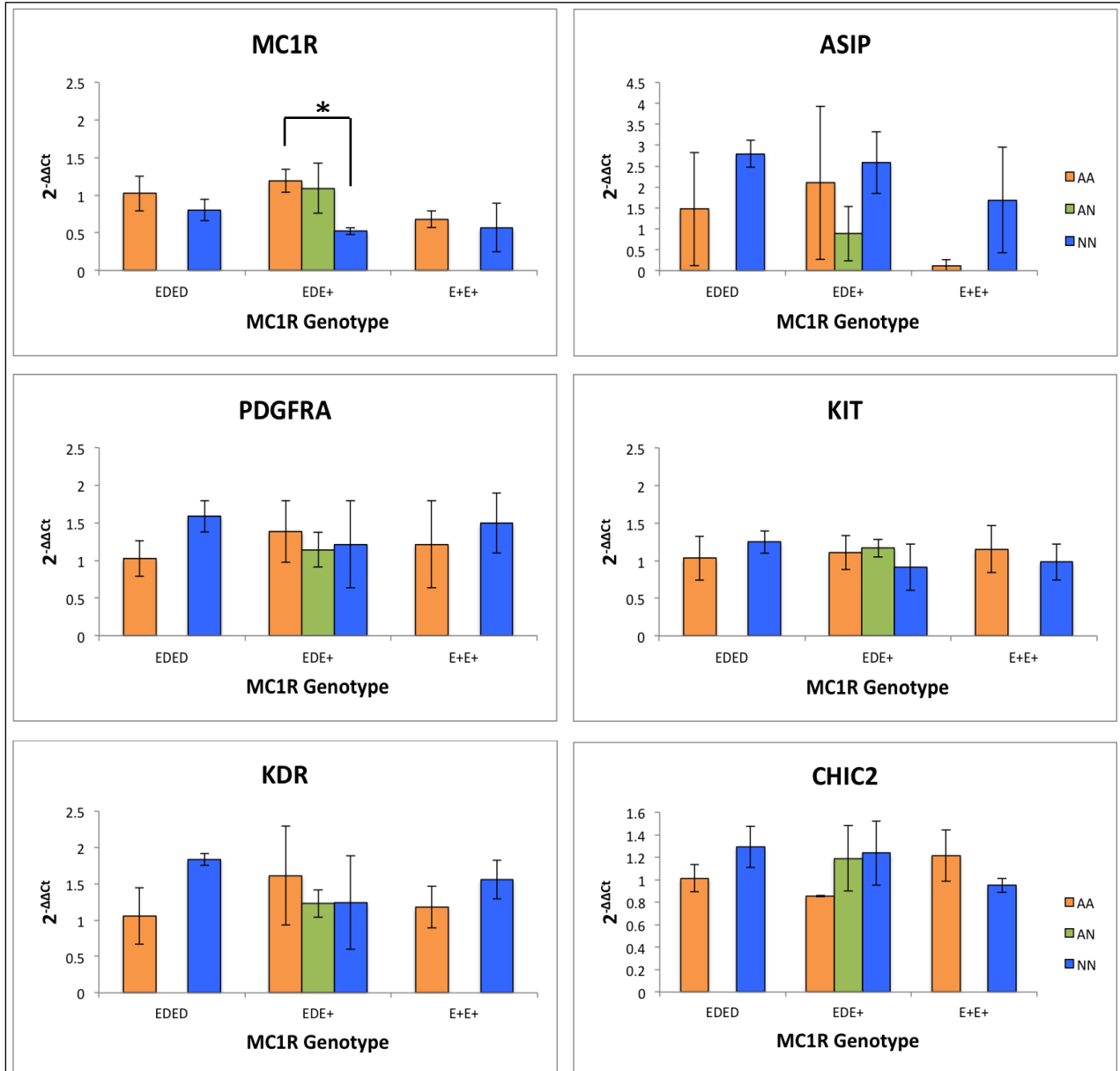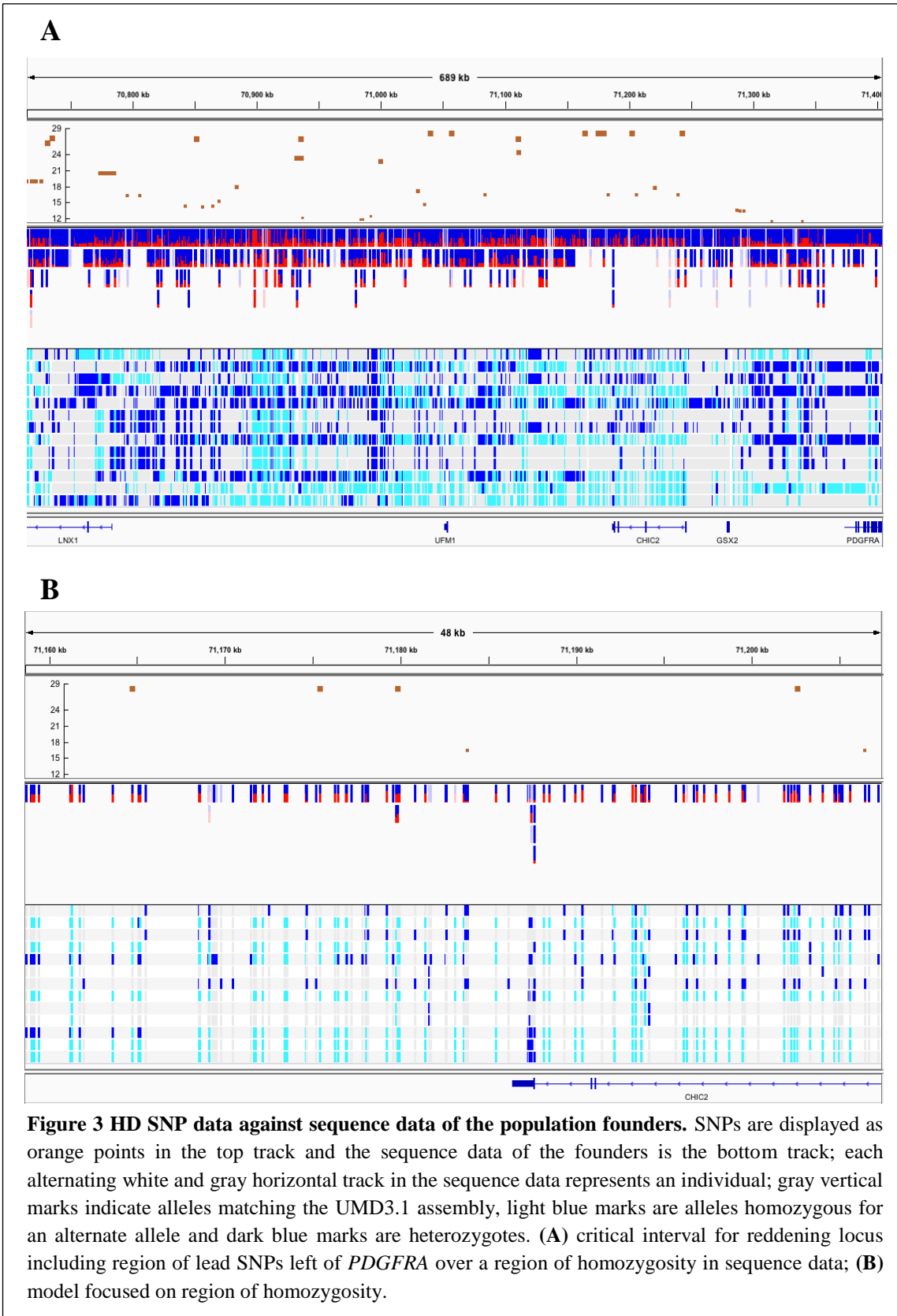y their extension locus genotype, then again (shown by the colored bars) by their reddening locus genotype; each of the colored bars are the mean relative expression for the biological triplicates and the standard deviation within the triplicates is designated by the error bars.

whereas higher levels of *MC1R* are seen for the homozygous Angus individuals. No significant differences in expression of the candidate genes (*PDGFRA*, *KIT*, and *KDR*) categorized by genotype at *MC1R* and reddening were found suggesting that none of these genes are the reddening locus.

Because we anticipate that the reddening allele will produce detectable differences in expression in skin, we reexamined the HD SNP data against the sequence data spanning the critical interval for the founders to identify other candidate genes. Careful examination of the region showed that the lead SNPs in the region were shifted to the left of *PDGFRA*. High amounts of heterozygosity were observed in the sequence data to the left and right of a small region of high homozygosity (Figure 2). This small, almost completely homozygous, region fell partly within the gene *CHIC2* (cysteine-rich hydrophobic domain-containing protein 2). None of these SNPs changed the coding sequence, which would appear to mean that if *CHIC2* is the causative gene then reddening is a regulatory effect. Relative expression of *CHIC2* was determined using the methods outlined previously for the other five target genes and the results are displayed in (Figure 3). No significant differences in expression were observed; however, the individuals possessing a Nellore allele at reddening and at least one dominant allele at extension tended to exhibit higher levels of expression for *CHIC2* when compared to the homozygous Angus individuals at reddening.

Figure 3 HD SNP data against sequence data of the population founders. SNPs are displayed as orange points in the top track and the sequence data of the founders is the bottom track; each alternating white and gray horizontal track in the sequence data represents an individual; gray vertical marks indicate alleles matching the UMD3.1 assembly, light blue marks are alleles homozygous for an alternate allele and dark blue marks are heterozygotes. **(A)** critical interval for reddening locus including region of lead SNPs left of *PDGFRA* over a region of homozygosity in sequence data; **(B)** model focused on region of homozygosity.

CHAPTER IV

DISCUSSION

Presence of an almost completely homozygous region partly within the gene *CHIC2* has led to investigation into the gene and its potential affects in melanogenesis. Although we are not aware of any previously documented role of *CHIC2* in pigmentation, it has been revealed to have involvement in a chromosomal translocation of PDGFRA t(4;12)(q11;p13) occurring in acute myeloid leukemia [16]. Through the detection of this translocation, Cools et al. [17] was able to isolate and overexpress the gene in *C. elegans* leading to the identification of the CHIC family consisting of two genes, *CHIC1* and *CHIC2*. Results of the overexpression study illustrated the localization of *CHIC2* at the plasma membrane and vesicular structures [17]. These genes were renamed from Brx and BTL after the discovery of a highly conserved cysteine-rich hydrophobic stretch of 23 amino acids, consisting of 8 cysteine residues, that was termed the CHIC motif [17]. Cools et al. found that the CHIC motif resembled the cysteine string domain of the cysteine string proteins (CSPs), a class of palmitoylated proteins present on vesicular membranes. CSPs have been implicated in regulated exocytosis in mammalian neuroendocrine and endocrine cell types through evidence of its ability to chaperone the conformational folding of components of the vesicular exocytotic machinery [18]. Cools et al. concluded that the extent of palmitoylation of the cysteines determines their membrane association and with the gene having a similar structure to CSPs, it is presented as a potential new family involved with vesicular transport.

Furthermore, palmitoylation involves the attachment of palmitate, a 16-carbon saturated fatty acid, to cysteine residues of proteins causing pleiotropic effects on protein function [19]. In

regards to signal transduction via G protein, the combination of palmitoylation, prenylation and myristoylation to a protein is involved in connecting the G protein to the plasma membrane to interact with its receptor [19]. *MC1R* is a member of the G protein-coupled receptor (GPCR) family located in the plasma membrane of melanocytes and having seven transmembrane domains. Through extracellular signals, the receptor's proteins will initiate binding of the G-protein to the guanine nucleotide binding site enabling an extracellular ligand, such as α-MSH, to exert a specific effect into the cell [20, 21]. We suspect *CHIC2* to be potentially disrupting the binding of G protein and subsequent binding of α-MSH to result in an abundance of pheomelanin synthesis and different degrees of reddened cattle. Implication of *CHIC2* in vesicular transport also leads to the potential for failed transport of eumelanin out of the melanocyte.

Although, no significant differences in expression of the candidate genes (*PDGFRA*, *KIT*, and *KDR*) categorized by genotype at *MC1R* and reddening were found, long-range regulatory effects on *PDGFRA*, *KIT* or *KDR* still cannot be ruled out. Geissler et al. [22] reports *KIT* to have a critical function in melanoblast migration during embryonic development. *KDR* has been more so implicated as a vascular endothelial growth factor (VEGF) receptor with functions in angiogenesis and high expression in melanomas [23]. Based on these data *PDGFRA* remains a positional candidate with reported roles in gastrulation signaling [9] and in the development of neural-crest derived pigment cells, including proliferation, migration, and tissue remodeling [10]. As well as possible influence in the mitogen activated protein kinase (MAPK) signaling pathway in melanogenesis and therefore the expression of MITF, a downstream regulator known to control the cascade of melanogenesis [1]. Even with the identification of *CHIC2* as an additional

positional candidate, analysis of expression data from skin failed to reveal any significant direct

effects of the gene on *MC1R*.

CHAPTER V

CONCLUSION

A critical interval on BTA6 most likely to contain the reddening locus was refined from BTA6: 71,708,424 –72,478,577 using Btau 4.0 assembly coordinates to BTA6: 70,714,167 – 71,404,818 now based on UMD3.1 coordinates. Although, no significant differences in expression of the candidate genes (*PDGFRA*, *KIT*, and *KDR*) categorized by genotype at *MC1R* and reddening were found, long-range regulatory effects on *PDGFRA*, *KIT* or *KDR* still cannot be ruled out. High density SNP data identified *CHIC2* as another strong positional candidate gene for reddening; however, analysis of expression data from skin failed to reveal any significant direct effects of the gene on *MC1R*. This study evaluated relative expression levels within skin biopsies of cattle, which included dermis and hair follicles. Further research using only hair follicles will be required to distinguish patterns of expression seen.

REFERENCES

1. Hulsman Hanna *et al.*: **Identification of a major locus interacting with *MC1R* and modifying black coat color in an F$_2$ Nellore-Angus population.** *Genetics Selection Evolution* 2014 **46:**4.

2. Klungland H, Våge DI, Gomez-Raya L, Adalsteinsson S, Lien S: **The role of melanocyte-stimulating hormone (MSH) receptor in bovine coat color determination.** *Mamm Genome* 1995, **6**:636–639.

3. Robbins LS, Nadeau JH, Johnson KR, Kelly MA, Roselli-Rehfuss L, Baack E, Mountjoy KG, Cone RD: **Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function.** *Cell* 1993, **72**:827–834.

4. Graphodatskaya D, Joerg H, Stranzinger G: **Molecular and pharmacological characterization of the MSH-R alleles in Swiss cattle breeds.** *J Recept Signal Transduc Res* 2002, **22**:421–430.

5. Olson TA: **Genetics of colour variation.** *The Genetics of Cattle* (1999): 33-53.

6. Klungland H, Olsen HG, Hassanane MS, Mahrous K, Våge DI: **Coat colour genes in diversity studies.** *Journal of Animal Breeding and Genetics* 2000, **117**: 217–224.

7. Silvers WK: **The agouti and extension series of alleles, umbrous, and sable**. *The Coat Colors of Mice, A Model for Mammalian Gene Action and Interaction*. W.K. Silvers, ed. (New York Springer-Verlag) 1979, pp. 6-44.

8. Hearing V, Jiménez M: **Mammalian tyrosinase – the critical regulatory control point in melanocyte pigmentation**. *International Journal of Biochemistry* 1987, **19**:1141-1147.

9. Morrison-Graham K, Schatteman GC, Bork T, Bowen-Pope DF, Weston JA: **A PDGF receptor mutation in the mouse (Patch) perturbs the development of a non-neuronal subset of neural crest-derived cells**. *Development* 1992, **115**: 133-142.

10. Andrae J, Gallini R, Betsholtz C: **Role of platelet-derived growth factors in physiology and medicine**. *Genes & Dev* 2008, **22**: 1276-1312.

11. Yipeng Fan, Peng Wang, Weixuan Fu, Tian Dong, Chao Qi, Lin Liu, Gang Guo, Cong Li, Xiaogang Cui, Shengli Zhang, Qin Zhang, Yi Zhang, Dongxiao Sun: **Genome-wide association study for pigmentation traits in Chinese Holstein population**. *Anim Genet* 2014, **45**: 740-744.

12. Fontanesi L, Tazzoli M, Russo V, Beever J: **Genetic heterogeneity at the bovine KIT gene in cattle breeds carrying different putative alleles at the spotting locus**. *Anim Genet* 2009, **41**:295–303.

13. Scheet P, Stephens M: **A fast and flexible statistical model for large-scale population genotype data: applications to inferring missing genotypes and haplotypic phase.** *The American Journal of Human Genetics* 2006, **78**:629-644.

14. Brannan J, Holman P, Olafson P, Pruett J, Riggs P: **Evaluation of methods for the isolation of high quality RNA from Bovine and Cervine hide biopsies.** *Journal of Parasitology* 2013, **99**:19-23.

15. Livak K, Schmittgen T: **Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method.** *Methods* 2001, **25**:402-408.

16. Fink S, Belongie K, Paternoster S, Smoley S, Pardanani A, Tefferi A, Van Dyke D, Ketterling R: **Validation of a new three-color fluorescence in situ hybridization (FISH) method to detect CHIC2 deletion, FIP1L1/PDGFRA fusion and PDGFRA translocations.** *Leukemia Research* 2009, **33**:843-846.

17. Cools J, Mentens N, Marynen P: **A new family of small, palmitoylated, membrane-associated proteins, characterized by the presence of a cysteine-rich hydrophobic motif.** *FEBS Letters* 2001, **492**:204-209.

18. Chamberlain L, Burgoyne R: **Cysteine string protein functions directly in regulated exocytosis.** *Mol. Biol. Cell* 1998, **9**:2259-2267.

19. Resh M: **Palmitoylation of Ligands, Receptors, and Intracellular Signaling Molecules.** *Science Signaling* 2006, **2006**:re14.

20. Sullivan KA, Miller RT, Masters SB, Beiderman B, Heideman W, Bourne HR: **Identification of receptor contact site involved in receptor–G protein coupling.** *Nature* 1987, **330**:758-760.

21. Fredriksson R, Lagerström MC, Lundin L, Schiöth HB: **The G-Protein-Coupled Receptors in the Human Genome Form Five Main Families. Phylogenetic Analysis, Paralogon Groups, and Fingerprints.** *Molecular Pharmacology* 2003, **63**:1256-1272.

22. Geissler EN, Ryan MA, Housman DE: **The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene.** *Cell* 1988, **55**:185–192.

23. Straume O, Akslen LA: **Expresson of Vascular Endothelial Growth Factor, Its Receptors (FLT-1, KDR) and TSP-1 Related to Microvessel Density and Patient Outcome in Vertical Growth Phase Melanomas.** *Am J Pathol.* 2001, **159**:223–235.