

Genetic polymorphisms in bovine *ferroportin* are associated with beef iron content

by

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS	iii
ACKNOWLEDGMENTS	iv
ABSTRACT	vi
CHAPTER 1 GENERAL INTRODUCTION	1
Thesis organization	1
Review of literature	1
I. Iron and health	1
II Factors affecting iron content	4
Environmental and physiological factors	4
Genetic factors	6
III. Iron homeostasis	8
Intestinal iron absorption	8
Iron utilization	10
Regulation of iron homeostasis	11
Iron-loading disorders	13
IV. Ferroportin	14
General description	14
Ferroportin regulation	15
Ferroportin-linked iron-loading disorder	19
V. Concluding remarks	20
CHAPTER 2 GENETIC POLYMORPHISMS IN BOVINE <i>FERROPORTIN</i> ARE	
ASSOCIATED WITH BEEF IRON CONTENT	24
Abstract	25
Introduction	26
Materials and Methods	28
Results	31
Discussion	34
Acknowledgements	39
References	39
GENERAL SUMMARY	51
Summary of results and general conclusion	51
Future work	53
LITERATURE CITED	56

LIST OF ABBREVIATIONS

BMP:	bone morphogenetic protein
Cp:	ceruloplasmin
Cybrd1:	duodenal cytochrome b (also known as DCYTB)
DMT1:	divalent metal transporter 1
ESCRT:	endosome sorting complex required for transport
Fe-S cluster:	iron-sulfur cluster
Fpn:	ferroportin
FTH1:	ferritin heavy chain
FTL:	ferritin light chain
<i>HAMP</i> :	hepcidin gene
HCP1:	heme carrier protein 1
HFE:	human hemochromatosis protein
HH:	hereditary hemochromatosis
IREs:	iron responsive elements
IRPs:	iron regulatory proteins
LD:	longissimus dorsi
Steap3:	six-transmembrane epithelial antigen of the prostate 3
SNPs:	single nucleotide polymorphisms
TfR1:	transferrin receptor 1
TfR2:	transferrin receptor 2
TMPRSS6:	transmembrane protease, serine 6
MVB:	the multivesicular body
UTR:	untranslated region

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ABSTRACT

Iron is an essential element for almost all living organisms. Tissue iron concentration shows natural variations among individuals, because of the influence of both environmental and genetic factors. Body iron, though essential, is also toxic in excess by generating reactive oxygen species. Iron homeostasis, thus, must be maintained systemically by the rate of iron absorption, iron utilization, iron storage and the rate of iron recycling. It is possible that mutations in any of the genes that encode proteins involved in maintaining iron homeostasis have the potential to alter iron load. The effect of single nucleotide polymorphisms (SNPs), a major genetic factor, on beef iron content were investigated in the current study.

The first objective of this study was to determine the variation of iron content in bovine longissimus dorsi (LD) muscle. The second objective was to identify single nucleotide polymorphisms (SNPs) in the exons and their flanking regions of the bovine *ferroportin* gene (*Fpn*) and to evaluate the association between the identified SNPs and bovine muscle iron content. LD muscle samples were collected from 1086 Angus cattle for iron quantification and genomic DNA extraction. Nine exons and their flanking regions of *Fpn* were amplified and sequenced with 6 selected DNA samples. Genotyping was carried out for 1086 cattle. Nine novel SNPs, NC007300: g.1780 A>G, g.1872 A>G, g.7169 C>T, g.7477 C>G, g.19208 C>T, g.19263 A>G, g.19427 A>G, g.19569 C>T and g.20480 C>T, were identified. Statistical analysis showed that three of the nine SNPs, g.19208 C>T, g.19263 A>G, and g.20480 C>T, were significantly ($P < 0.007$) associated

with muscle iron content. High linkage disequilibrium was observed for SNP g.19208 C>T, g. 19263 A>G and g. 20480 C>T ($R^2 > 0.99$), with which two haplotypes, TGC and CAT, were defined. Beef from individuals that were homozygous for the TGC haplotype had significantly ($P < 0.001$) higher iron contents than did beef from CAT homozygous or heterozygous individuals.

In conclusion, results of the current study indicated that SNPs, NC007300: g.19208 C>T, g.19263 A>G, and g.20480 C>T, in *Fpn* might be useful markers for the selection of Angus cattle that produce progeny with a more desirable iron composition and therefore improve the healthfulness of beef. Further studies are needed to verify the observed effect in other independent populations and elucidate the biological mechanisms of the SNP effects.

CHAPTER 1

GENERAL INTRODUCTION

Thesis organization

This thesis is to partially fulfill the requirement of the degree of Master of Science. It is organized as a general literature review followed by one complete manuscript for submission to Animal Genetics. The review of literature introduces the background of the topics addressed in the manuscript in the main body of the thesis. The manuscript consists of seven sections, an abstract, introduction, materials and methods, results, discussion, acknowledgements and references. It is formatted according to the requirement of the journal. The manuscript is preceded by a general summary section including summary of results and general conclusion and future work. Then, a reference section provides the literature cited in the literature review and general summary section.

Review of literature

I. Iron and health

Iron is an essential element for almost all living organisms. It functions as a cofactor for a variety of proteins in diverse biochemical processes. Iron, for example, is a vital component of heme in hemoglobin, myoglobin, and cytochromes (Edison et al., 2008). It is also an important element in iron-sulfur (Fe-S) clusters that can serve as excellent

electron donors and acceptors participating in electron transfer, such as in mitochondrial respiratory complex I – III. Moreover, Fe-S clusters function in enzyme catalysis and in sensing environmental or intracellular conditions to regulate gene expression, such as the post-transcriptional regulation by Fe-S clusters of iron regulatory protein 1 (IRP1; Lill, 2009). Here and throughout this review of literature, iron will refer to the ionic form of iron in living organisms rather than elemental iron unless mentioned.

As a redox-active transition metal, iron, however, also can be toxic at high concentration by generating reactive oxygen species (Andrews and Schmidt, 2007; Valko et al., 2005). Hence, organisms have developed a tight regulatory mechanism of iron homeostasis to face the challenge of obtaining adequate amounts of iron to facilitate biological processes and yet avoid the toxicity associated with free iron (Andrews and Schmidt, 2007).

From a nutrition point of view, both iron deficiency and overload have great consequences and epidemiological significance (Cairo et al., 2006). Iron deficiency anemia, with an estimated 3 billion people affected, is still a major worldwide public health problem (Andrews, 2008). Hereditary hemochromatosis (HH), an iron overload disorder, is one of the most common genetic disorders in Caucasian population. It is also one of the first human genetic diseases to be linked to a discrete chromosomal position (Andrews, 2008). Several mutations in genes that encode proteins involved in maintaining iron homeostasis have been shown to be associated with HH (Table 1).

In healthy adults, daily iron loss is estimated to be 1 mg in men and 1.5 mg in women, which is the result of the exfoliation, blood loss, or sweat. It can be compensated

by the absorption of 1-2 mg iron daily from diet (Zhang et al., 2009). Dietary iron usually presents in two forms: inorganic non-heme iron and organic heme iron.

Dietary non-heme iron is present in a wide variety of foodstuffs. It is free or in weak complexes with other food components such as phytate or tannins during digestion (Theil, 2004). In contrast, heme iron is in a stable porphyrin complex, whose pyrrole structure protects the iron against the interference from iron chelators such as polyphenols and phytate, which results in more efficient absorption compared with that of non-heme iron (Andrews, 2005).

Heme iron is primarily from animal sources in the form of hemoglobin or myoglobin. Beef is a good source of dietary iron for humans and other animals in regard to both amount and bioavailability. It is known to be the richest source of iron compared with lamb, pork, chicken, and turkey (Carpenter and Clark, 1995). A 100 g portion of raw beef can contribute to more than 38% of the daily iron requirement of an adult (Giuffrida-Mendoza et al., 2007). In addition, the absorption of beef iron is efficient because of the high heme to non-heme iron ratio. Besides the high bioavailability of beef iron itself, beef consumption can enhance the absorption of dietary non-heme iron through the presence of “meat factor”, which may be attributed to the partially digested peptides from muscle proteins. These peptides were found to bind iron via their cysteine and histidine residues to form complexes that were soluble and available for absorption (Hurrell et al., 2006). Two-fold higher non-heme iron absorption was observed from meals that contained beef compared with the control meals with egg albumin (Cook and Monsen, 1976).

II Factors affecting iron content

Meat exhibits natural variations in the amounts of nutrients contained therein. This variability could be the result of environmental and physiological factors such as age, muscle type, gender, species, and breed. It also could be the result of genetic factors such as single nucleotide polymorphisms (SNPs; Greenfield, 2003).

Environmental and physiological factors

Age Iron content of muscle increases with age. Doornenbal and Murray (1981) found a significant positive effect ($P < 0.001$) of age on muscle iron content with 20 mature, purebred Shorthorn cows ranging in age from 3 to 12 years. Rincon-Villalobos and Huerta-Leidenz (2007) confirmed the age effect on beef longissimus dorsi thoracis iron content in 64 water buffalo and 68 Zebu-influenced cattle in four age groups (7, 17, 19, and 24 months of age). Besides that, they also observed age by species and age by gender interactions that contributed to the variation of beef iron content, which ranged from 1.74 to 2.56 mg/100 g of wet weight.

Muscle type Mineral concentrations vary between different muscle types as a result of their different intensity of physical activities and the effects of muscle fiber types (Lin et al., 1989). Doornenbal and Murray (1981) determined iron concentration in three types of muscle—the longissimus dorsi, semimenbranosus, and diaphragm muscle—among 96 animals with an age range of 421-492 days. Iron content was significantly different among the three muscle types. Longissimus dorsi had significantly lower iron

concentration than did the other two, whereas diaphragm had the highest iron content.

Gender Beef iron concentration has not been reported to be influenced by gender, though several other minerals were found to be affected, including calcium, zinc, magnesium, sodium, and potassium (Doornenbal, 1981). Gender effect on iron content, however, has been reported in pork. Wiseman and his colleagues (2007) determined iron content of pork in two genders at various body weight intervals from 20 to 125 kg. Iron content was found to be greater in gilts than in barrows.

Breed of sire Similar to that of gender, the effect of breed is not pronounced for beef iron content. Other minerals such as calcium, zinc, and potassium were significant different among breeds (Doornenbal, 1981). Consistent with the result found in cattle, another study showed no difference of the iron content in longissimus dorsi muscle in two breeds of goat (Park, 1988).

Diet The effect of dietary iron on beef iron content is significant. Variation in the total iron concentrations of beef was observed for groups of calves fed different amount of dietary iron. Muscle heme pigment concentrations also were found to be different at slaughter (Miltenburg et al., 1992). According to Shenk et al. (1934), grass or grass plus grain feeding can increase the myoglobin content of beef compared with feeding only grain.

Interaction between elements Interactions between elements affect the mineral concentration in beef adversely. The interactions has been found between cadmium and zinc and between copper and iron in hens, ruminants, and humans (Anke et al., 1970).

The changes caused by the interactions could be explained by the functions of some metal

transporters on the surface of epithelial cells that do not show exclusive substrate preferences. Divalent metal transporter 1 (DMT1) is known as an iron transporter protein that imports iron into the cell, whereas manganese also is considered to be a potential substrate for the same transporter. In a rat model, a mutation, which rendered DMT1 ineffective, not only caused severe anemia but also decreased tissue manganese concentration (Chua and Morgan, 1997). Hansen et al. (2009) investigated the effects of dietary iron on manganese absorption in 24 weaned pigs fed three different amounts of iron supplementation. They found duodenal manganese concentrations were greater in low iron treatment group than that in high iron treatment group. Their result suggested a competition between iron and manganese for DMT1. Recently, the same research group reported that growing beef calves with severe deficiency of copper not only showed decreased iron status but also showed alterations in the expression of certain iron regulatory genes which encoded hepcidin, ferroportin, and intestinal ferritin (Hansen et al., 2010).

Genetic factors

Single nucleotide polymorphisms (SNPs) are considered to be the most common polymorphisms and potentially responsible for most of the phenotypic variations present (Vignal et al., 2002). A SNP is a DNA sequence variation that occurs when a single base change with a typical alternative of two possible nucleotides at the given position (Weller, 2001).

Genetic mutations can give rise to two types of SNPs: purine-purine or

pyrimidine-pyrimidine exchanges that are called transitions or pyrimidine-purine or purine-pyrimidine exchanges that are called transversions. Observed data showed a bias for SNPs towards the transitions (Collins and Jukes, 1994). SNPs may be found within the coding sequences of genes, non-coding sequences of genes, or in the intergenic regions between genes. A SNP in a coding sequence of a gene does not necessarily affect the amino acid sequence of the produced protein, because of the degeneracy of the genetic code. Then, the SNP is termed as synonymous mutation (also called silent mutation). Otherwise, it is called non-synonymous mutation. SNPs that are not in protein-coding regions, especially those in the 5' or 3' untranslated regions (UTRs), may still have consequences for exon recognition during pre-mRNA splicing and mRNA stability.

SNPs are very useful as molecular markers. A SNP identified in a functional gene region may likely be the causal mutation or the source of phenotypic variation (Salisbury et al., 2003). In addition, SNPs are present throughout many genomes at a high frequency (Carlson et al., 2001; Konfortov et al., 1999)

Genetic influence on iron content variation in human is indicated by inherited disorders such as hereditary hemochromatosis (HH), which is characterized by systemic iron overload. Most cases of HH in humans are believed to be the result of homozygosity for the C282Y mutation in the hemochromatosis gene (*HFE*), which encodes the human hemochromatosis protein (HFE). As most quantitative characteristics with a genetic component seem to be affected by multiple genes, Whitfield et al. (2000) did an association assessment in a human cohort study to compare the effect of *HFE*

polymorphism with other genetic factors in body iron variation. Their results suggested significant effects from other unknown genes on iron storage. They also provided evidence for the necessity of a further search for polymorphisms in iron homeostasis related genes. Mutations in any of the genes which encode proteins involved in iron regulation, iron absorption, iron utilization, and iron storage have the potential to alter iron load (Constantine et al., 2009). In different strains of inbred mice, SNPs in the promoters of hepcidin were found to be correlated with iron-loading variations (Bayele and Srai, 2009). In human cohort studies, serum iron status has been found to be significantly associated with SNPs in the genes encoding bone morphogenetic protein 2 (BMP2; Milet et al., 2007), duodenal cytochrome b (Cybrd1; Constantine et al., 2009), transferrin (Tf; Benyamin et al., 2009b), and transmembrane protease, serine 6 (TMPRSS6; Benyamin et al., 2009a).

III. Iron hemeostasis

Intestinal iron absorption

Body iron is mainly obtained from the diet. The iron absorption takes place primarily in the proximal portion of the duodenum. Normally, there is little or no paracellular iron transport. Iron, thus, must be transported across both the apical and basolateral membranes of the enterocytes to enter the circulation (Andrews, 2008).

Most dietary non-heme iron is in the bio-unavailable ferric (Fe^{3+}) form, which must be reduced to ferrous (Fe^{2+}) form for transport. This process is facilitated by brush border

ferrireductase (Figure 1). The first intestinal ferrireductase to be identified is duodenal cytochrome b (Cybrd1; McKie et al., 2001). However, Cybrd1 knock-out mice did not show any apparent disorder, which suggested the existence of other mechanisms for iron reduction (Gunshin et al., 2005b). Later, another ferrireductase— six-transmembrane epithelial antigen of the prostate 3 (Steap3) was found, which is important for transferrin (Tf)-transferrin receptor 1 (TfR1)-mediated iron uptake in erythroid cells.

DMT1 serves as the primary transmembrane iron transporter (Gunshin et al., 1997), bringing the reduced Fe^{2+} into the enterocytes. The crucial role of DMT1 has been confirmed by targeted mutation of the murine *DMT1* gene (Gunshin et al., 2005a). Once the iron enters the epithelial cells, a large portion is transported across the basolateral membrane of the enterocytes, which is considered as absorbed. A small portion is retained in the enterocytes and stored in ferritin, a major iron storage protein. The retained iron is lost during enterocytes senescence and sloughed into the gut. Ferroportin (Fpn) is currently the only known protein responsible for cellular iron export (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). Iron is exported in Fe^{2+} form, which needs to be oxidized to Fe^{3+} form before binding to serum transferrin. The oxidation of Fe^{2+} to Fe^{3+} is facilitated by multicopper ferroxidases including serum protein ceruloplasmin (Cp) and membrane-bound intestinal hephaestin (Cherukuri et al., 2005; Vulpe et al., 1999). Cp is also found to be necessary for maintaining Fpn activity on the cell surface (De Domenico et al., 2007a).

Though the absorption of non-heme iron is understood in some detail, that of heme iron, which is mainly from animal source, remains uncertain (Andrews, 2008). Recently,

heme uptake was found to be mediated by heme carrier protein 1 (HCP1) which is localized on the brush-border membrane in the proximal duodenum (Shayeghi et al., 2005). It is proposed that, after heme is transported into the enterocytes by HCP1, iron is liberated from the protoporphyrin ring by heme oxygenase and joins the same intracellular pathway as non-heme iron (Andrews, 2005).

In the circulation system, nearly all absorbed iron bind rapidly to transferrin (Tf), an abundant extracellular protein with high iron-binding affinity (Cheng et al., 2004). Tf keeps iron nonreactive and soluble, delivering it through circulation and extravascular fluid to tissues for utilization or storage.

Iron utilization

The largest user of iron is the erythroid bone marrow. Tf-mediated endocytosis via TfR1 is considered to be the most important way for iron uptake in developing erythroid precursors. The acidic environment in the endosome leads to iron release from Tf. Released Fe^{3+} is reduced to Fe^{2+} by Steap3 and then transported into the cell by DMT1 (Hentze et al., 2004). Although TfRs are ubiquitously expressed, most non-hematopoietic tissues can assimilate iron without the TF cycle (Andrews and Schmidt, 2007).

Once iron is inside the cell, it can be directed towards mitochondria where the critical steps of heme synthesis and Fe-S cluster biogenesis take place or it can be stored in ferritin, an cellular iron storage protein. Heme synthesis mainly takes place in mitochondria, with the intermediate steps occurring in the cytosol (Ponka, 1997). Mitogerrin serves as the mitochondrial iron importer (Shaw et al., 2006).

Skeletal muscle is another large user of iron. Human skeletal muscle represents about 40% of body mass and contains around 10% to 15% of body iron, which is mainly in myoglobin. However, little is known about the iron assimilation in skeletal muscle (Robach et al., 2007).

Regulation of iron homeostasis

Iron is essential for the fundamental aspect of cellular function, but it is also toxic in excess by generating reactive oxygen species. Iron homeostasis, therefore, needs to be maintained meticulously from the process including iron transport, utilization, and storage. Regulatory mechanisms have been identified for keeping cellular and systemic iron balance (Hentze et al., 2004).

At the cellular level, one of the regulatory mechanisms is through ferritin, a ubiquitous iron storage protein. Ferritin acts as a buffer which can bind iron at iron overload and allow for iron mobilization when needed (Theil, 2003). The other protective mechanism is post-transcriptional regulation through the interaction between iron responsive elements (IREs) and iron regulatory proteins (IRPs). IREs are stem and loop structures localized in the 5' or 3' UTRs of mRNAs. It can be recognized and bound by IRPs at low iron condition. During iron overload, IRP2 is ubiquitinated and degraded (Iwai et al., 1998), whereas IRP1 is assembled with a Fe-S cluster, which prevents IRE binding and converts IRP1 to a cytosolic aconitase (Rouault et al., 1991). Depending on the location of IREs, the interaction between IRPs and IREs serves different roles (Pantopoulos, 2004; Pantopoulos and Hentze, 1998). IRE/IRP complexes formed in the 5'

UTR of an mRNA inhibit translation. These IREs has been identified for the mRNAs encoding ferritin heavy and light chains (FTH1 and FTL), erythroid 5-aminolevulinic acid synthase, mitochondrial aconitase, and Fpn. (Muckenthaler et al., 1998). A second type of IREs is located at the 3' UTR of mRNA encoding TfR1. IRP-binding can stabilize the mRNA through protecting it against the nuclease digestion (Hentze and Kuhn, 1996). The interaction between IRPs and IREs is regulated by the cellular iron pool as well as some other factors such as reactive oxygen species (Pantopoulos and Hentze, 1998).

Iron homeostasis need also to be maintained systemically throughout the body. Systemic iron homeostasis involves the need to balance among intestinal iron absorption, iron utilization, iron recycling, and iron storage (Andrews, 2008).

Hepcidin, a peptide hormone produced by hepatocytes, is mainly responsible for modulating systemic iron homeostasis (Nicolas et al., 2001). Hepcidin is a 25-amino-acid protein that primarily is secreted by the liver but also is produced in heart, pancreatic, and hematopoietic cells (Peyssonnaud et al., 2006). Its importance is established by several observations: 1. Hepcidin expression increases when excess iron is administered (Pigeon et al., 2001); 2. Forced expression of a hepcidin transgene causes anemia (Nicolas et al., 2002); and 3. Targeted mutation of *HAMP* (the hepcidin gene) causes severe iron overload (Viatte et al., 2005). It is reported that hepcidin regulates intestinal iron absorption and macrophage iron release by the control of cellular iron export through Fpn (Nemeth et al., 2004). *In vitro* studies showed that hepcidin can bind directly to cell surface Fpn, inducing Fpn internalization and ubiquitin-mediated degradation (De Domenico et al., 2007b). In the small intestine, inactivation of Fpn leads to the retention

of iron in the intestinal epithelium without absorption. In the macrophage where iron recycling and storage take place, removal of Fpn from the cell surface causes interrupted release of iron (Andrews, 2008).

The transcription of *HAMP* is activated by the bone morphogenetic protein (BMP)/SMAD pathway. Liver-specific disruption of SMAD4 led to significant decrease of hepcidin expression and iron accumulation in many organs (Wang et al., 2005). In addition, Babitt et al. (2007) showed BMP2 could positively regulate hepcidin expression *in vivo*. Recently, BMP6 was identified as a master hepcidin regulator, which indicated a critical role of BMP6 in iron homeostasis (Meynard et al., 2009).

Iron-loading disorders

Iron-loading disorders mainly include iron deficiency anemia, hemochromatosis, and the anemia of chronic disease. Many of them are inherited iron disorders (Table 1).

Hereditary hemochromatosis (HH) is defined as an iron-loading disorder caused by a genetically determined failure to prevent unneeded dietary iron from entering the circulatory pool and is characterized by progressive hepatic parenchymal iron overload with the potential for multiorgan damage and disease (Pietrangelo, 2006). It is usually inherited in an autosomal-recessive pattern, with a high prevalence up to 1 in 100 individuals in northern European populations (Merryweather-Clarke et al., 2000). Little was known about the molecular basis of HH until 1996 when Feder and his colleagues (1996) discovered a mutation (C282Y) in hemochromatosis gene (*HFE*) in the majority of hemochromatosis patients. Till now, mutations have been identified in many other genes

that encode proteins involved in iron homeostasis.

The molecular pathogenesis of HH can be divided into three categories. The first one is the mutations in *HAMP*, disturbing the production of functional hepcidin protein. Hepcidin, with critical importance in the regulation of iron homeostasis, also is considered to play a central role in the pathogenesis of HH (Roetto et al., 2003). The second category involves the mutations in the genes encoding HFE (*HFE*; Feder et al., 1996), TfR2 (*TfR2*; Camaschella et al., 2000), and hemojuvelin (*HFE2*; Papanikolaou et al., 2004), resulting in depressed hepcidin expression. The last group is the mutations in the *Fpn* gene, which cause Fpn insensitive to hepcidin regulation or Fpn subcellular mislocalization and thus lead to HH (Montosi et al., 2001; Njajou et al., 2001).

IV. Ferroportin

General description

Ferroportin (Fpn, also known as IREG1, and MTP1), which was discovered simultaneously by three different research groups, is the only currently known cellular iron exporter protein found in vertebrates (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). It shares no homology with either iron importer DMT1 or other mammalian proteins. Ferroportin is a multipass integral membrane protein. A topological structure model provided by Rice et al. (2009) shows Fpn with 12 transmembrane domains and both N and C termini in the cytoplasmic side. The oligomeric state of Fpn is an issue still under debate. Evidence from different sources indicates the possible

existence of monomers, dimers, or multimers.

Fpn is reported to be present in all cell types that export ferrous iron including enterocytes, macrophages, white blood cells involved in erythrophagocytosis, Kupffer cells, brain astrocytes, and placental cells. In addition, Fpn is highly expressed on the surface of cells with high iron export capacity, especially in enterocytes and tissue macrophages (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000).

Fpn plays a crucial role as the only cellular iron exporter. Evidence from *Fpn* knockout mouse study showed an early failure in embryonic development, suggesting the importance of Fpn in the transport of iron from extra-embryonic visceral endoderm prior to placental formation. Intestine-specific inactivation of Fpn resulted in severe iron deficiency, which confirmed the essentiality of Fpn in the intestinal iron absorption (Donovan et al., 2005).

Ferroportin regulation

Ferroportin expression is controlled by two regulatory mechanisms: post-transcriptional regulation by IRP/IRE interaction and post-translational regulation by hepcidin.

Ferroportin mRNA is reported to bear a functional IRE motif in its 5' UTR (McKie et al., 2000). Overexpression of *Fpn* in tissue culture cells resulted in IRP1 activation (Abboud and Haile, 2000). The binding of IRP to IREs at the 5' UTR blocks the initiation of translation by interfering with ribosome assembly at the start codon. This IRP/IRE regulation ensures that the expression of Fpn is repressed in low iron conditions to

maintain cellular iron balance (Leipuviene and Theil, 2007).

Galy et al (2008) provided evidence for the significance of IRP-mediated post-transcriptional control of Fpn expression in the duodenum. In mice lacking intestinal IRP expression, they found a great enhancement of Fpn expression under normal mRNA abundance, accompanied with increased expression of negative regulator hepcidin. Recently, Zhang et al. (2009) reported a Fpn transcript that was expressed by utilizing an alternative upstream promoter in duodenal epithelial and erythroid precursor cells. This transcript of Fpn lacked the 5' IREs and was not repressed in low iron conditions, which suggested a way for Fpn to bypass IRP-dependent regulation.

Besides translational regulation by IRP/IRE interaction, Fpn expression also is controlled by hepcidin-mediated post-translational regulation, which is also the major mechanism for maintaining whole-body iron homeostasis. Hepcidin is a peptide hormone, which is primarily produced by hepatocytes and secreted into the circulation system. The human *HAMP* gene encodes an 84-residue prepropeptide with a 24-residue N-terminal signal peptide, which is under subsequential cleavage to produce pro-hepcidin. Mature hepcidin is a 25-amino-acid peptide that is processed from pro-hepcidin. Mass spectrometry and chemical analysis showed 8 cysteine residues in the peptide, all in disulfide bonds, which suggested a highly constrained structure of hepcidin (Park et al., 2001). Mutations of *HAMP* led to systemic iron overload or hemochromatosis (Nemeth et al., 2004). Hepcidin binds directly to Fpn, triggering Fpn phosphorylation, internalization, and consequently ubiquitin-mediated degradation. The removal of Fpn from cell surface blocks iron efflux and decreases serum iron concentration. Fpn is the major protein

through which hepcidin regulates serum iron abundance and tissue iron distribution (Nemeth et al., 2004).

Phosphorylation and ubiquitination are two protein modifications known to signal the internalization of membrane proteins (Bonifacino and Traub, 2003). Phosphorylation of Fpn is the primary event in response to hepcidin binding, which occurs rapidly on the cell surface. It is a critical signal for clathrin-coated pits mediated internalization. Two adjacent tyrosine residues, Y302 and Y303, were identified to be the phosphorylation site on Fpn. Mutation of both tyrosine residues to phenylalanine prevented hepcidin-mediated Fpn internalization but did not affect Fpn localization (De Domenico et al., 2007b). In addition, the src kinase inhibitor inhibited hepcidin-mediated tyrosine phosphorylation, internalization, and degradation of Fpn, which indicated that the tyrosine kinase that phosphorylated Fpn belonged to the src kinase family. The topological structure of Fpn has not been conclusively resolved, and it remains to be determined what conformational change occurs during Fpn and hepcidin interaction. Several working models have been proposed. One model proposed by Liu et al. (2005) shows that the two tyrosines Y302 and Y303 are located between transmembrane regions VI and VII, facing the cytoplasm, whereas another model provided by Rice et al. (2009) places the two tyrosines in the middle of transmembrane region VI, which is not directly accessible to cytosolic kinases. They suggested a conformational shift during the binding of hepcidin to the extracellular loop of Fpn, which exposes the tyrosine residues to the cytosol for phosphorylation.

Ubiquitination of Fpn takes place subsequently to internalization that is required for trafficking and degradation. Lysine residue K253 on the cytosolic side of Fpn was found

to be the site for ubiquitination. Mutation of K253 resulted in a protein with no ubiquitination that was degraded at a much slower rate than was the wide type, even though the mutant protein was properly targeted to the cell surface, phosphorylated, and internalized in response to hepcidin (De Domenico et al., 2007b). According to the model proposed by Liu et al. (2005), the identified ubiquitination site is on the same cytosolic loop as the phosphorylation sites, which suggests a relationship between ubiquitination and phosphorylation. Fpn is trafficked to the lysosome after ubiquitination. This process depends on the multivesicular body (MVB) pathway and requires participation of all three endosome sorting complex required for transport (ESCRT) complexes and late-acting accessory factors (De Domenico et al., 2007b).

In summary, the regulation of Fpn involves the coordination of both the IRE/IRP network and the hepcidin regulatory system. IRP-controlled post-transcriptional regulation of Fpn reflects cellular iron balance, whereas the post-translational regulation performed by hepcidin reflects systemic iron requirements (Muckenthaler et al., 2008). Moreover, though there is no known regulatory mechanism for iron excretion, regulation of iron transport through Fpn in intestinal enterocytes implicates an explanation. Body iron deficiency increases Fpn expression, which allows for increased intestinal absorption and recovery of iron from intestinal epithelium. On the other hand, body iron overload decreases Fpn expression, thus increases the amount of iron retained in enterocytes, which is lost into the lumen during enterocyte exfoliation (Donovan et al., 2005).

Ferroportin-linked iron-loading disorder

Ferroportin-linked iron loading disorder is referred to as type IV HH (“ferroportin disease”). The first mutation related to this disease was identified simultaneously by two research groups in 2001 (Montosi et al., 2001; Njajou et al., 2001). Till now, several Fpn mutants have been identified (Figure 2), all of which cause missense changes and exert their disease phenotype in an autosomal dominant fashion. Some mutations were found to develop hyperferritinaemia with high ferritin but low transferrin saturation and mostly macrophage/Kupffer cell iron loading (Pietrangelo, 2004), whereas others have abnormalities similar to typical HH with high transferrin saturation, and hepatocyte iron loading (Sham et al., 2005).

Based on the molecular basis of the ferroportin disease, the mutants with heterogenous phenotypes as mentioned above can be divided into two categories. One group shows loss of iron export function, including Fpn mutant $\Delta 162$, $\Delta 160-162$, G323V, and G490D. The defect in the iron export function is resulted from the subcellular mislocalization of Fpn, even though the protein expression profile is similar to that of the wild type. The mutant Fpn, instead of localizing to the cell surface, was found to be primarily intracellularly trapped in the endoplasmic reticulum. The lack of membrane expression of Fpn results in the loss of iron export function and also the loss of interaction with hepcidin. This reduction in cellular iron efflux is reflected by the high ferritin in cells and iron overload in tissues that require the largest iron flows (i.e., macrophages) In contrast, the second group of Fpn mutants retained full iron export activity but does not

appropriately respond to hepcidin regulation. For example, mutant N144H shows resistance to hepcidin regulation. This mutant Fpn has no hepcidin-induced degradation, even though hepcidin binding is not altered. It is suggested that N144H mutation might disrupt the Fpn domain that is required for internalization and degradation. On the other hand, another mutant Q182H shows normal membrane localization and hepcidin-induced degradation, but the internalization was delayed. More detailed mechanism remains to be characterized (De Domenico et al., 2005).

V. Concluding remarks

During the past decade, knowledge in understanding mammalian iron transport and its regulation has substantially increased. Many key proteins involved in regulating iron homeostasis have been identified and their molecular functions, though still under intensive study, have been partially elucidated. A combination of insights into cellular iron balance and systemic iron homeostasis will facilitate our understanding of the signaling mechanism of local and whole-body management of iron need. Fpn is of great interest because of its critical role as the only known cellular iron exporter and as the receptor for hepcidin, which reflect the coordination between cellular iron balance and systemic iron homeostasis. All the progress made will lead to a better understanding of the molecular basis and hereditary nature of iron disorders, potentially contributing to future therapeutic advances.

Table 1. Genes involved in inherited human iron disorders (Adopted from Andrews, 2008).

Protein (gene symbol)	Protein function	Disease (<i>caused by loss-of-function mutations unless otherwise noted</i>)
Ceruloplasmin (Cp)	Plasma ferroxidase	Aceruloplasminemia
DMT1 (SLC11A2)	Transmembrane iron importer	Anemia with hepatic iron overload
Ferritin H chain (FTH1)	Subunit of iron storage protein; has ferroxidase activity	Iron overload (<i>Mutation disrupting iron regulatory element</i>)
Ferritin L Chain (FTL)	Subunit of iron storage protein	Hyperferritinemia-cataract syndrome (<i>mutation disrupting iron regulatory element</i>)
Ferroportin (SLC40A1)	Transmembrane iron exporter	Macrophage-predominant iron overload (<i>Loss-of-function mutation that cause protein mislocalization</i>) Hemochromatosis (<i>gain-of-function mutation that cause insensitivity to hepcidin</i>)
Frataxin (FXN)	Mitochondrial iron chaperone	Friedreich ataxia
Glutaredoxin 5 (GLRX5)	Participates in Fe-S cluster biogenesis	Anemia with iron overload and sideroblasts
Hemojuvelin (HFE2)	Bone morphogenetic protein coreceptor	Juvenile hemochromatosis
Hepcidin (HAMP)	Iron regulatory hormone, binds ferroportin to cause its inactivation and degradation	Juvenile hemochromatosis
HFE (HFE)	Regulates hepcidin expression, mechanism uncertain; interacts with TFR1 and TFR2; may participate in a signaling complex with TFR2	Classic HLA-linked hemochromatosis
Mitoferrin (SLC25A37)	Mitochondrial iron import	Erythropoietic protoporphyria
Transferrin (TF)	Plasma iron binding protein, ligand for TFR1 and TFR2	Atransferrinemia (<i>hypotransferrinemia</i>)
Transferrin receptor-2 (TFR2)	Sensor for diferric transferrin; regulates hepcidin expression; may participate in a signaling complex with HFE	Hemochromatosis

Figure 1. A generic mammalian cell depicted to illustrate cellular iron import, utilization, and export (Adopted from Hentze et al., 2004).

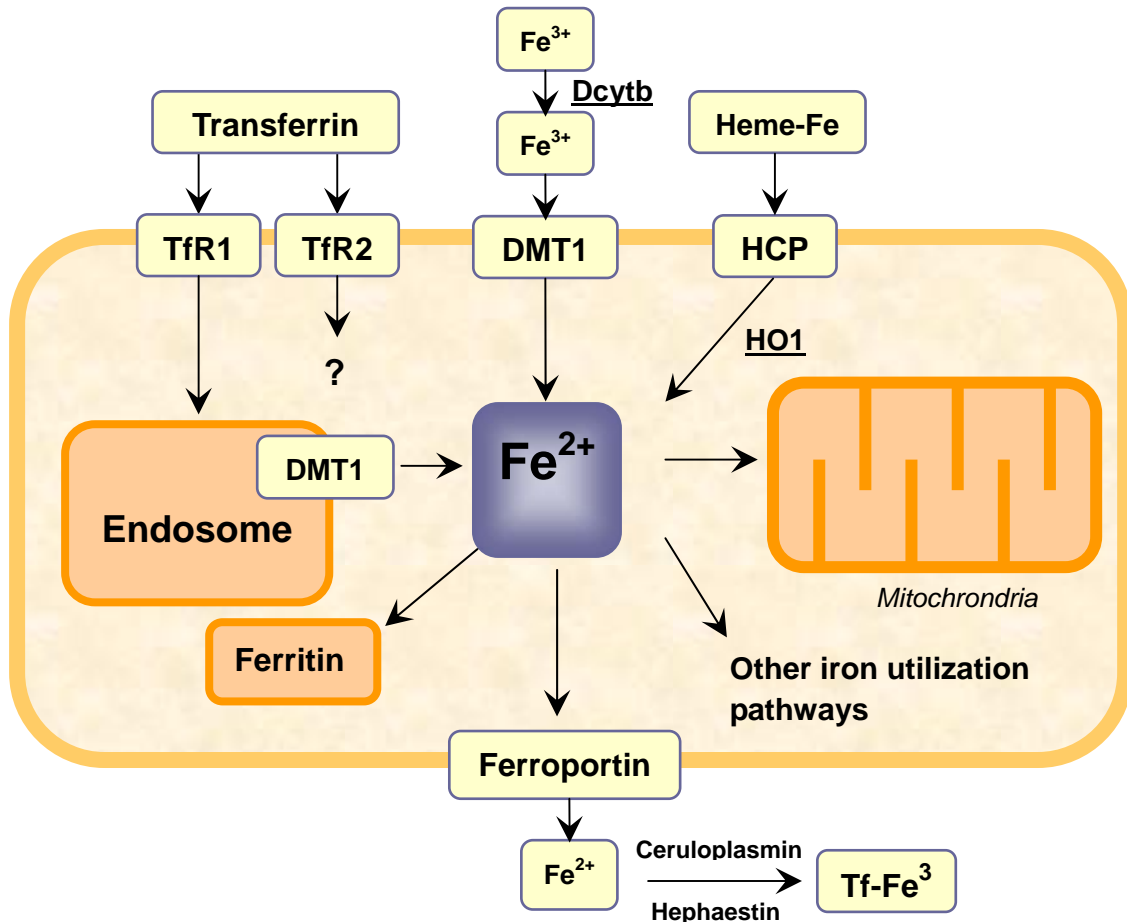
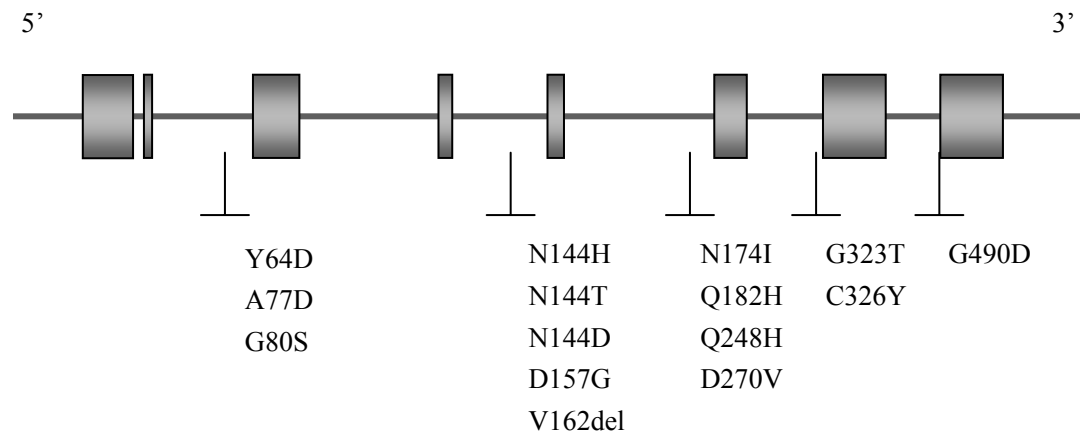


Figure 2. Mutations in human *Fpn* gene (Adopted from Le Gac and Ferec, 2005).



CHAPTER 2

GENETIC POLYMORPHISMS IN BOVINE *FERROPORTIN* ARE ASSOCIATED WITH BEEF IRON CONTENT¹

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Abstract

We hypothesized that genetic polymorphisms in *ferroportin* (*Fpn*), which encodes the only known cellular iron exporter, could influence muscle iron content. The objective of this study was to identify single nucleotide polymorphisms (SNPs) in the exons and flanking regions of the bovine *Fpn* gene and to evaluate the extent to which they were associated with beef iron content. Longissimus dorsi (LD) muscle samples were collected from 1086 Angus cattle for iron quantification and DNA extraction. All exons and their flanking regions of *Fpn* were amplified and sequenced with 6 selected DNA samples for SNP identification. Genotyping with the identified SNPs were carried out for the 1086 cattle. Nine novel SNPs, NC007300: g.1780 A>G, g.1872 A>G, g.7169 C>T, g.7477 C>G, g.19208 C>T, g.19263 A>G, g.19427 A>G, g.19569 C>T and g.20480 C>T, were identified, among which SNP g.19208 C>T, g.19263 A>G, and g.20480 C>T were significantly ($P < 0.007$) associated with muscle iron content. High linkage disequilibrium was observed for SNP g.19208 C>T, g.19263 A>G, and g.20480 C>T ($R^2 > 0.99$) with which two haplotypes, TGC and CAT, were defined. Beef from individuals that were homozygous for the TGC haplotype had significantly ($P < 0.001$) higher iron content than did beef from CAT homozygous or heterozygous individuals. In conclusion, SNPs, NC007300: g.19208 C>T, g.19263 A>G, and g.20480 C>T might be useful markers for the selection of Angus cattle that produce progeny with a more desirable iron composition. Further studies are needed to verify the observed effect in other independent populations and elucidate the biological mechanism of the SNP effects.

Introduction

Iron, an essential element, is required as an ionic cofactor for a variety of proteins and functions as an excellent biological electron donor and acceptor (Edison et al., 2008; Lill, 2009). However, iron deficiency anemia, with an estimated 3 billion people affected, is still a major worldwide public health problem (Andrews, 2008). Beef is a good source of dietary iron in regard to both amount and bioavailability (Purchas et al., 2003).

As a redox-active transition metal, iron, though important physiologically, is toxic at high concentration by generating reactive oxygen species (Galaris et al., 2008). Iron homeostasis, thus, must be maintained systemically by the rate of iron absorption through the duodenal mucosa, the rate of iron release from storage, and the rate of iron utilization. There is no paracellular iron transport under normal circumstances. So iron needs the assistance of transporter proteins to cross the cell membrane. Among several identified iron transporters, ferroportin (Fpn) is the only known iron exporter, which facilitates iron transport out of a cell (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). In addition, Fpn is the receptor of hepcidin, a peptide hormone that acts as the central regulator of serum iron concentration. Binding of hepcidin to Fpn leads to Fpn internalization and degradation and consequently decreases the iron efflux into the plasma (De Domenico et al., 2007). Evidence from *Fpn* knockout mouse study showed an early failure in embryonic development, which indicated an important role of Fpn in maternoembryonic iron transfer (Donovan et al., 2005). Intestine-specific inactivation of Fpn resulted in severe iron deficiency, which confirmed the essentiality of Fpn in the

intestinal iron absorption (Donovan et al., 2005). Fpn is reported to be highly expressed on the surface of skeletal muscle cells. The expression of muscle Fpn also is regulated by serum hepcidin concentration (Robach et al., 2009).

Iron content in beef is known to vary with physiological and environmental factors, such as animal age, muscle type (Doornenbal, 1981), diet (Shenk et al., 1934) and interaction between elements (Anke et al., 1970). Influence from genetic factors is indicated by inherited disorders such as hereditary hemochromatosis (HH) which is characterized by systemic iron overload (Le Gac and Ferec, 2005). Most cases of HH in human were believed to be the result of polymorphisms in hemochromatosis gene (*HFE*). However, it appears that most quantitative characteristics with genetic components are affected by multiple genes. Whitfield et al. (2000) compared the effect of *HFE* polymorphisms and other genetic factors on the variation of iron stores and showed considerably greater effects from other unknown genes on iron stores in addition to *HFE*. It is suggested that mutations in any of the genes encoding proteins in iron regulation, iron absorption, iron utilization, and iron storage have the potential to alter iron load (Constantine et al., 2009). Till now, several proteins have been reported to be associated with HH besides *HFE*, including hemojuvelin, transferrin receptor-2 (TfR2), and Fpn. Mutations in human *Fpn* gene cause systemic iron overload, which is referred to as type IV hereditary hemochromatosis (“ferroporotin disease”; Cazzola, 2003; Pietrangelo, 2004). Besides the disease-related mutations, more than six hundred SNPs, mostly in human and mice, have been identified in the coding and non-coding regions of *Fpn*. To our knowledge, no SNPs have been reported for *Fpn* in cattle yet. Moreover, skeletal

muscle, which contains 10 to 15% of body iron in human, is of great interest regarding iron homeostasis (Robach et al., 2007). However, little is currently known about the genetic factors that may influence iron storage in cattle, especially in skeletal muscle.

Growing evidence shows that polymorphisms in genes that encode a component of the iron regulation network potentially can influence whole-body iron content. In addition, *Fpn* plays an important role in the maintenance of iron homeostasis. Therefore, we hypothesized that variation in *Fpn* gene among individuals would be a candidate for heritable differences in iron content of skeletal muscle and might be used as markers to improve the healthfulness of iron content in beef while maintaining other positive physical and chemical attributes of the food product.

In this study, we identified nine novel single nucleotide polymorphisms (SNPs) mostly in the flanking regions of bovine *Fpn* exons. We then evaluated the relationship between *Fpn* genotype and iron content of LD muscle in Angus cattle and observed significant associations between the three SNPs and the iron content of beef.

Materials and Methods

Animals and sample collection

Angus cattle (n=1086) were used in this study. The cattle consist of 391 young bulls, 181 steers and 154 heifers from Iowa State University Angus breeding project (Ames, Iowa) and 360 steers from collaborators in California. All of the cattle were raised with no implants and no antibiotic treatment. They were harvested at commercial

facilities with an average age of 457 ± 46 days. Longissimus dorsi muscle samples were collected, trimmed of external connective tissue and adipose tissue, freeze ground, packed, and stored at -20°C until iron concentration analysis.

Iron concentration analysis

Beef samples were dried, and moisture was determined according to AOAC official method 934.01 (2005). Dry samples were subjected to closed-vessel microwave digestion process (CEM, MDS-2000) with 5 mL concentrated nitric acid and 2 mL 30% hydrogen peroxide according to AOAC official methods 999.10 (Jorhem and Engman, 2000). Microwave programs were set as following: 250 watts for 5 min, 630 watts for 5 min, 500 watts for 20 min, and 0 watts for 15 min. Total iron concentration in beef was determined by using inductively coupled plasma-optical emission spectroscopy (ICP-OES, SPECTRO Analytical Instruments).

DNA polymorphism identification and genotyping

Genomic DNA was purified by phenol chloroform method. Six DNA samples, half from cattle with high muscle iron and half from cattle with low muscle iron, were selected for SNP identification. Eight pairs of PCR primers were designed to amplify the nine exons and their adjacent intronic regions of *Fpn* gene (Table 1). The product sizes for the eight sets of primers were 965, 1000, 927, 677, 799, 799, 831, and 682 bp, respectively. The PCR mixture contained 12.5 ng genomic DNA, 500 nM of each primers, 5 μL GoTaq[®] colorless master mix (1.5 mM MgCl_2 , 0.2 mM dNTP mixture, and GoTaq[®] DNA

polymerase; Promega) at a final volume of 10 µL. The PCRs were performed in a DNA engine thermal cycler (Bio-Rad) with the following protocol: 94 °C for 5 min; followed by 39 cycles of 94 °C for 30 s; 58 °C for 35 s, and 72 °C for 35 s; with a final extension step at 72 °C for 5 min. PCR products were purified using ExoSAP-IT® (USB). The DNA sequences of PCR amplicons were determined with ABI 3730 DNA Analyzer (Applied Biosystems Inc.) at the Iowa State University DNA Facility. The genotypes of the identified SNPs in the 1086 Angus cattle were determined by Sequenom® at Genomic Technologies Core Facility at Iowa State University.

Statistical analysis

Data were analyzed by using a mixed linear model (PROC MIXED; SAS Inst., Inc.) according to the following statistical model:

$$Y_{ijklm} = \mu + S_i + C_j(S_i) + A_k + G_l + G_l \times S_i + \text{Sire}_m + e_{ijklm},$$

Where,

Y_{ijklm} = dependent variable (beef iron concentration);

μ = overall mean;

S_i = fixed effect of the i th level of source ($i = 1, 2$);

$C_j(S_i)$ = fixed effect of the j th level of contemporary group nested with in i th level of source ($j = 1 - 19$);

A_k = age as covariance of the k th observation ($k = 1 - 1086$);

G_l = Fixed effect of the l th level of genotype in the genotype as class effect model ($l = 1, 2, 3$),

or

genotype (homozygote AA = -1, heterozygote AB = 0, and homozygote BB = 1) as covariance of the l th observation in the allele substitution model;

$G_l \times S_i$ = interaction term of the l th genotype with the i th source;

$Sire_m$ = random effect of sire of observation m ; $sire_m \sim N(0, \sigma_s^2)$;

e_{ijklm} = random error term; $e_{ijklm} \sim N(0, \sigma_e^2)$.

Significance threshold correction for multiple comparisons was determined with the method developed by Cheverud (2001). After adjustment, P values that were less or equal to 0.007 were determined to be significant, and P values that were between 0.007 to 0.014 were classified as statistical tendencies. Least square means (\pm SE) were determined by using the genotype as class model as defined above. Mean values were compared by using pairwise t-tests. Additive genetic effect of each locus was estimated as the difference between the two homozygous groups. The dominance effect was estimated as the difference between the heterozygous group and the average of the two homozygous groups in each locus (Khatib et al., 2007). The haplotype and linkage disequilibrium were analyzed by using Haploview (Barrett et al., 2005).

Results

DNA polymorphism identification and selected nucleotide sequence alignment

Bovine *Fpn* is located on chromosome 2. The gene is 23,683 bp in length, with nine exons. We amplified and sequenced the nine exons of *Fpn* and their flanking regions

of six Angus cattle. Nine nucleotide substitutions were identified, which were NC007300: g.1872 A>G, g.7169 C>T, g.7477 C>G, g.19208 C>T, g.19263 A>G, g.19427 A>G, g.19569 C>T, and g.20480 C>T. Two SNPs, g.19427 A>G and g.20480 C>T were located in the exons. SNP g.19427 A>G in exon 7 was predicted to result in an amino acid replacement from methionine (ATG) to valine (GTG) and the other one, g.20480 C>T in exon 8, was a synonymous mutation.

The rest of the SNPs were in intronic regions, four of which were located close to exons. Polymorphism g.7169 C>T was -6 nt from the beginning of exon 4, SNP g.19208 C>T and g.19263 A>G were located -80 and -26 nt upstream of exon 7, respectively. SNP g.19569 C>T was +36 nt downstream of exon 7. Nucleotide sequence alignments were carried out by using BLAST (<http://www.ncbi.nlm.nih.gov/blast>) for g.7169 C>T, g.19208 C>T, g.19263 A>G, and g.20480 C>T among cattle, human, chimpanzee, monkey, house mouse, and rat (Figure 1). All of the four polymorphisms were found to be conserved among these species and also in conserved noncoding or coding sequences.

Genotype frequencies of the identified SNPs

Angus cattle (n = 1086 head) were genotyped with the nine identified SNPs. Most of the SNPs were in Hardy-Weinberg Equilibrium, except g.7169 C>T and g.19263 A>G. SNP g.1780 A>G, g.1872 A>G, g.7169 C>T, and g.7477 C>G showed a low minor allele frequency that is less than 3%, in which g.1780 A>G showed a rare allele with frequency less than 1% (Table 2). For SNP g.19208 C>T, g.19263 A>G, g.19427 A>G, g.19569 C>T, and g.20480 C>T, approximately half of the cattle were heterozygous.

Nearly complete linkage disequilibrium was observed among SNP g.19208 C>T, g.19263 A>G, and g.20480 C>T ($R^2 > 0.99$), which defined two haplotypes, TGC and CAT, with frequency 61.1% and 38.6%, respectively (Figure 2).

Association of SNP genotype with iron content of LD muscle

With the genotype result, we tested for the association between the nine SNPs identified and muscle iron content. Five SNPs were found to have P -value less than 0.05. After adjustment for multiple comparisons, P values that less than 0.007 are considered to be statistically significant. Three SNPs, g.19208 C>T, g.19263 A>G, and g.20480 C>T, were strongly ($P < 0.007$) associated with iron content after the adjustment both in the genotypic as class effect model and allele substitution model. Polymorphism g.7477 C>G, tended ($P < 0.014$) to be associated with iron content in the genotypic as class effect model (Table 3). No significant associations were detected between the iron content and SNP g.1780 A>G, g.1872 A>G, g.7169 C>T, g.19427 A>G, and g.19569 C>T. Similar results were obtained for both cattle from Iowa and those from California. As a result, we only present the combined analysis.

Three genotypes were significantly ($P < 0.005$) associated with high beef iron content compared with other genotypes. They were TT genotype in 19208, GG genotype in 19263 and CC genotype in 20480.

Moreover, as mentioned above, SNP g.19208 C>T, g.19263 A>G, and g.20480 C>T were in almost complete linkage disequilibrium ($R^2 > 0.99$) and defined two haplotypes, TGC and CAT (Table 4). Homozygote with TGC or CAT haplotype had frequency of

39.87% or 16.40%, respectively, and the frequency for the heterozygote was 43.73%. The association analysis between the haplotypes and beef iron content showed that beef from individuals that were homozygous for the TGC haplotype had significantly ($P < 0.001$) higher iron contents than did beef from CAT homozygous or heterozygous individuals.

Contribution of *Fpn* genotype to iron content of LD muscle

Additive and dominant effect were tested for haplotype TGC and CAT in SNP g.19208 C>T, g.19263 A>G, and g.20480 C>T (Table 4). Significant ($P < 0.005$) additive effect was observed for this haplotype block.

Discussion

Fpn is the only identified cellular iron exporter that plays an important role in maintaining iron homeostasis. Currently, over 600 SNPs have been reported for mainly human and mice in the coding and non-coding regions of *Fpn*, but none for cattle. In this study, nine novel SNPs, NC007300: g.1780 A>G, g.1872 A>G, g.7169 C>T, g.7477 C>G, g.19208 C>T, g.19263 A>G, g.19427 A>G, g.19569 C>T, and g.20480 C>T, were identified in the exons and their flanking regions of *Fpn*. Comparison between the SNPs identified in this study and the known SNPs of *Fpn* in NCBI SNP database showed no similarity.

In addition, strong associations between the identified SNPs and beef iron content were found in this study. Among the identified SNPs, g.19208 C>T, g.19263 A>G, and g.20480 C>T were associated significantly ($P < 0.007$) with the iron content. SNP g.7747

C>T tended ($P < 0.014$) to be associated with LD muscle iron content. With the growing understanding of the molecular basis of iron regulation, several mutations have been reported in human *Fpn* gene that link with iron overload disorders, which is referred to as type IV hereditary hemochromatosis (“ferroporotin disease”; Cazzola, 2003; Pietrangelo, 2004). All identified mutations in *Fpn* are missense mutations that lead to amino acid substitutions or deletions (De Domenico et al., 2005). Moreover, several studies either in human or in mice were conducted to relate variation in body iron status with polymorphisms in iron-related genes. In human cohort studies, serum iron status was found to be associated significantly with polymorphisms in genes that encode bone morphogenetic protein 2 (*BMP2*; Milet et al., 2007), duodenal cytochrome b (*CYBRD1*; Constantine et al., 2009), transferrin (*TF*; Benyamin et al., 2009b), and transmembrane protease, serine 6 (*TMPRSS6*; Benyamin et al., 2009a). In different strains of inbred mice, SNPs in the promoter region of hepcidin were found to be correlated with iron-loading difference (Bayele and Srail, 2009). No association between iron status and *Fpn* has been reported yet. To our knowledge, this was the first study addressing the association between genetic polymorphisms in *Fpn* and variation of muscle iron content.

The three SNPs, g.19208 C>T, g.19263 A>G, and g.20480 C>T, were significantly ($P < 0.007$) associated with beef iron content were of great interest. They were in nearly complete linkage disequilibrium and defined two haplotypes.

SNP g.19208 C>T and g.19263 A>G were located -81 and -26 nt upstream of the exon 7, respectively. SNP g.20480 C>T, though located in coding region, was not predicted to cause amino acid replacement. The location of this SNP, however, was of

great significance, because it was in exon 8, which encodes an important domain of Fpn. In human, this domain bears the hepcidin binding sites (Residue 324-343), phosphorylation sites (Y301 and Y302), and ubiquitination site (K253). Binding of hepcidin to Fpn leads to Fpn phosphorylation, internalization, ubiquitination and degradation. (De Domenico et al., 2009; De Domenico et al., 2007). Nucleotide sequence alignment among several species showed that the three SNPs were in conserved sequences. It has been suggested that similarities in coding and non-coding sequences between divergent organisms imply functional constraint. Therefore, the result from the nucleotide sequence alignment may indicate certain functional role for SNP g.19208 C>T, g.19263 A>G, and g.20480 C>T.

The efficiency of mRNA splicing site recognition is a combinatorial control of several parameters, such as splice site strength, the presence or absence of splicing enhancers and silencers, RNA secondary structures, and the exon/intron architecture. Mutations that affect these parameters potentially may influence pre-mRNA processing and consequently protein expression (Hertel, 2008). Exonic splicing enhancers (ESEs) are *cis*-acting RNA sequence elements located within exons that can increase exon inclusion by serving as binding sites for the assembly of multicomponent splicing enhancer complexes (Black, 2003). With online bioinformatics tool—ESE Finder (Cartegni et al., 2003; Smith et al., 2006), SNP g.20480 CC genotype was predicted to be in a putative alternative splicing factor/splicing factor 2 (ASF/SF2) binding site (GAGACGG), which was lost if the genotype was changed from C to T. Therefore, it is possible that SNP g.20480 C>T, though a synonymous mutation, would influence the function of Fpn via

the interference of the splicing efficiency of exon 8 as a part of ESE.

SNP g.19208 C>T and g.19263 A>G were located in the upstream close to exon 7. With an on-line alternative splice site predictor (Wang and Marin, 2006), SNP g.19263 A>G was found to be a few nucleotides away from a putative RNA splicing site. In addition, this polymorphism was not in Hardy-Weinberg equilibrium, which potentially indicates selection at this site. Mammalian RNA splicing is known to be regulated by *cis*-acting elements, either as enhancer or silencer. In human amyloid precursor protein gene, Shibata et al (1996) identified two *cis*-acting elements, ATGTTT and TTT, involved in the modulation of RNA splicing in the human *APP* gene. The two *cis*-elements found were in the upstream of exon 8, one as enhancer, and the other one as silencer. In the current study, the g.19208 locus was in a TTT sequence that might be involved in a *cis*-acting element, thereby potentially regulating the efficiency of the intron excision, whereas this explanation need to be taken cautiously, because the RNA splicing regulation may have tissue/cell-type specificity or depending on developmental stage (Pozzoli and Sironi, 2005). It is reported also that secondary structure may be part of the “mRNA splicing code” that determines exon recognition, though it remains unclear which motifs (loop or stem) are more favored by *trans*-acting factors (Hiller et al., 2007). As TTT is a stem-forming sequence, nucleotide replacement within this sequence would disrupt the secondary structure of pre-mRNA, which might consequently affect the following mRNA splicing.

Thus, the polymorphism in these loci might be able to influence the iron content of muscle via the interference of the efficiency of mRNA splicing recognition. Although

currently there is no evidence supporting the role for the three SNPs in *Fpn* expression, a possible involvement cannot be ruled out. High linkage disequilibrium observed for these three SNPs may be another explanation for the strong associations. It is also possible that they are in linkage disequilibrium with other causative mutations that have not been identified yet. Verification of the effect of these SNPs in other cattle populations is important to confirm the associations before they can be applied to marker-assisted selection.

SNP g.1780 A>G had low minor allele frequency of less than 1%. However, during the genetic association analysis, the only animals with g.1780 GG genotype was dropped off because of lacking sire information. Therefore, no association analysis had been done for g.1780 GG genotype. Future research is needed to investigate the effect of this rare allele on muscle iron content.

Besides SNP g.1780 A>G, SNP g.1872 A>G, g.7169 C>T and g.7477 C>G had also low minor allele frequency of 0.01, 0.03, and 0.01, respectively. The association analysis of these SNPs need to be interpreted cautiously because of the limited data on these genotypes as well as their large standard error.

Interestingly, polymorphism g.19427 A>G in exon 7, was predicted to cause codon change from ATG to GTG which consequently changed an amino acid from methionine to valine. However, the iron content of LD muscle was not statistically associated with this amino acid change. Sequence alignment performed among cattle, human, orangutan, chimpanzee, mouse, and rat showed that this site was conserved across all these species (data not shown).

In conclusion, nine novel SNPs in *Fpn*, NC007300: g.1780 A>G, g.1872 A>G, g.7169 C>T, g.7477 C>G, g.19208 C>T, g.19263 A>G, g.19427 A>G, g.19569 C>T, and g.20480 C>T, were identified in this study. SNP g.19208 C>T, g.19263 A>G, and g.20480 C>T are strongly associated with beef iron content, which might be useful markers for facilitating the selection of Angus cattle with more desirable LD muscle iron content and therefore to improve the healthfulness of beef. Further investigations are needed to verify the observed effect in other independent cattle populations and elucidate the biological mechanisms of the SNP effect.

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Table 1. Primer sequences.

Primer name	Sequence	
	Forward	Reverse
Fpn-1	TGAGGACTCCTTGATGACGA	ATGGGACAGGCAGTTACCAG
Fpn-2	GCCTTTCCAACCTTCAGCTACA	CACTAACACCTATGGGAAAACATC
Fpn-3	CTTGACATCCAAGGCATACTGA	TCCAATGCAAGTCCTGGTTA
Fpn-4	AATACGGTCGTTACGAATGG	GATCTGAAAGCTAGCAACTGGA
Fpn-5	CAGATGTGACCAAGCACTCA	ACCCACATCTCTCGCATCTC
Fpn-6	TCATGAAGATGGGAAGCGTA	GTTCAACCCAGATTTGTTCCA
Fpn-7	TGTGCCCTCCATAGGTGA	GCTGAATTATCTGACCCTCCA
Fpn-8	ACCTATCAATATATCAGGTCCGTTC	AGCAACAGCTGAACTGTCTTACAC

Table 2. Polymorphisms in *Fpn* exons and adjacent introns and genotype frequency.

Variable	g.1780 A>G			g.1872 A>G			g.7169 C>T		
	AA	AG	GG	AA	GA	GG	CC	CT	TT
Number of animals	479	31	1	802	237	15	25	216	779
Genotype frequency	0.94	0.06	0.00	0.76	0.23	0.01	0.03	0.21	0.76
Variable	g.7477 C>G			g.19208 C>T			g.19263 A>G		
	CC	CG	GG	CC	TC	TT	AA	GA	GG
Number of animals	16	272	824	157	486	412	161	457	437
Genotype frequency	0.01	0.25	0.74	0.15	0.46	0.39	0.15	0.43	0.42
Variable	g.19427 A>G			g.19569 C>T			g.20480 C>T		
	AA	AG	GG	CC	CT	TT	CC	CT	TT
Number of animals	139	362	234	117	266	157	377	503	167
Genotype frequency	0.19	0.49	0.31	0.22	0.49	0.29	0.36	0.48	0.16

Table 3. Single SNP genotypic and allelic association with beef iron content

SNP	Number of animals	<i>P</i> -value ^a for association test	
		Genotypic	Allelic
g.1780 A>G	470	0.4850	0.7986
g.1872 A>G	971	0.1056	0.0984
g.7169 C>T	935	0.0267	0.0118
g.7477 C>G*	1020	0.0133	0.0437
g.19208 C>T**	963	0.0003	< 0.0001
g.19263 A>G**	964	0.0022	0.0006
g.19427 A>G	662	0.9301	0.7509
g.19569 C>T	499	0.5665	0.6959
g.20480 C>T**	953	0.0016	0.0005

^a *P*-values were not corrected for multiple comparisons

* *P*-value < 0.014

** *P*-value < 0.007

Table 4. Effects of *Fpn* SNPs on LD muscle iron content.¹

Traits	g.1780 A>G			g.1872 A>G		
	AA	AG	GG	AA	AG	GG
Iron content	13.42 ± 0.21	13.54 ± 0.52	N.A.	13.25 ± 0.15	12.81 ± 0.21	13.39 ± 0.75
Traits	g.7169 C>T			g.7477 C>G		
	CC	CT	TT	CC	CG	GG
Iron content	13.55 ± 0.64	13.59 ± 0.22	13.03 ± 0.15	11.15 ± 0.74	13.04 ± 0.20	13.26 ± 0.14
Traits	g.19208 C>T			g.19263 A>G		
	CC	CT	TT	AA	AG	GG
Iron content	12.69 ± 0.25 ^a	12.92 ± 0.16 ^a	13.53 ± 0.17 ^b	12.79 ± 0.25 ^a	13.00 ± 0.17 ^a	13.54 ± 0.17 ^b
Traits	g.19427 A>G			g.19569 C>T		
	AA	AG	GG	CC	CT	TT
Iron content	13.20 ± 0.27	13.18 ± 0.21	13.10 ± 0.24	13.26 ± 0.29	13.58 ± 0.24	13.42 ± 0.29
Traits	g.20480 C>T					
	CC	CT	TT			
Iron content	13.54 ± 0.17 ^a	12.95 ± 0.16 ^b	12.77 ± 0.25 ^b			

¹ Values are expressed as least square means ± SE. Iron content are expressed as µg per gram of wet weight of longissimus dorsi muscle.

^{a, b} Values in each single nucleotide polymorphism (SNP) with different subscripts differ at $P < 0.005$.

Table 5. Effect of haplotypes of genotype g.19208 C>T, g.19263 A>G, and g.20480 C>T on LD muscle iron content and estimates (\pm SE) of additive and dominant effect associated with the haplotypes.

Genotype			Number of animals	Genotype frequency (%)	Iron content ¹
g.19208 C>T	g.19263 A>G	g.20480 C>T			
TT	GG	CC	372	39.87	13.51 ^A \pm 0.16
CT	AG	CT	408	43.73	13.00 ^B \pm 0.15
CC	AA	TT	153	16.40	12.80 ^B \pm 0.23
Haplotype additive effect			0.76 \pm 0.25***		
Haplotype dominant effect			-0.18 \pm 0.17		

¹ Values are expressed as LSmeans \pm SE. Iron content are expressed as μ g per gram of wet weight of longissimus dorsi muscle.

^{A, B} Values with different subscripts differ at $P < 0.001$.

*** $P < 0.005$.

Figure 1. Nucleotide sequence alignments for g.7169 C>T, g.19208 C>T, g.19263 A>G, and g.19569 C>T among several mammalian species. The multiple sequence alignments were carried out by using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The nucleotide polymorphism loci are shown in bold and in red.

g.7169 C>T

GTCAGCACCTTTTCTCCTGTCCTG T TTCCAGGGGGACCGGATGTGGCACT	7170	Cattle
ATCAAAACATTTTCT-CT-TTTCAT T TTA-AGGGAGATCGGATGTGGCACT	10487	Human
ATCAAAACATTTTCT-CT-TTTCAT T TTA-AGGGAGATCGGATGTGGCACT	8456	Chimpanzee
ATCAAAACATTTTCT-CT-TTTCAT T TTA-AGGGAGATCGGATGTGGCACT	9640	Monkey
AACCAAAACATCTTTC----TTT TG TTA-AGGGGGATCGGATGTGGCACT	4275	House mouse
AGCCAAACTTCTTTC----TCT TG TTA-AGGGGGATCGGATGTGGCACT	4613	Rat

g.19208 C>T

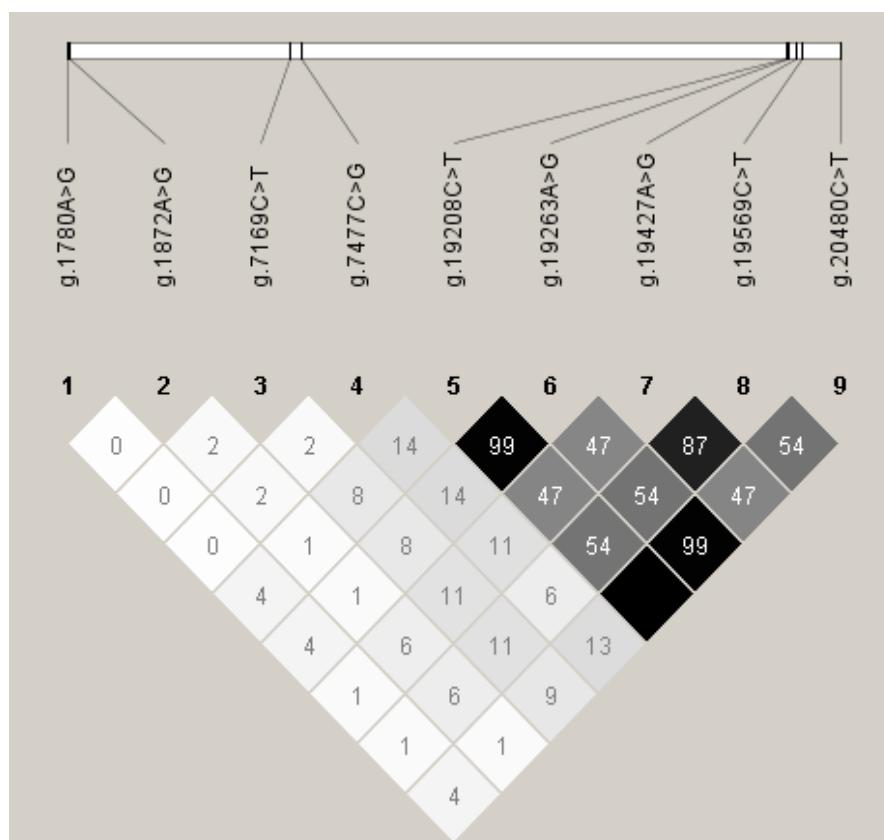
ACA-----GTTAATCAATACAC T TGGTTTA-TGAGTTGCGTTATGTAC	19241	Cattle
ATG-----TCTAATCTATACTC T TGGTTTACAGCTTTGTATTGTGTAA	20164	Human
ATGCAAAATGTCTAATCTATACTC T TGGTTTACAGCTTTGTATTGTATAA	18627	Chimpanzee
ATG-----GCTAATCTGTACTC T TGGTTTACAGGTTTGTATTGTGTAA	18768	Monkey
ATG-----GTTAGTCTGTACTG T TGGTTT--AGATTATAGCACATAA	13075	House mouse
ATG-----GATGGTCTGCACTG T TGGTTT--AGACTTATAGCACGTAA	13390	Rat

g.19263 A>G

TCTCTTTGATAGATTTGCACACTT G CCTGCCTTTTTACCTATCTCTGTA	19291	Cattle
TCTCTTTGATGGGTTTGCACACTT A CCTGCCTCTTTACCTGCCTCTCTA	20214	Human
TCTCTTTGATGGGTTTGCACACTT A CCTGCCTCTTTACCTGCCTCTCTA	18677	Chimpanzee
----TTTGATGGGTTTGTACACTT A CCTGCCTCTTTACCTGCTTCTCTA	18816	Monkey
TGTAACCTCT-----CACTT A CCTGCCTCTTGACCTACCTGTGTA	13115	House mouse
TTTCTTTCACAGTTTGAACGCTC A CTCTCCTCTTTACCTACTTC--TA	13438	Rat

g.20480 C>T

TGAGCCCTTCCGCACTTTCCGAGAC G GATGGGTCTCCTATTACAACCAGT	20505	Cattle
TGAGCCCTTCCGTACCTTCCGAGAT G GATGGGTCTCCTACTACAACCAGC	21742	Human
TGAGCCCTTCCGTACCTTCCGAGAT G GATGGGTCTCCTACTACAACCAGC	20200	Chimpanzee
TGAGCCCTTCCGTACCTTCCGAGAT G GATGGGTCTCCTACTACAACCAGC	20353	Monkey
AGAGCCCTTCCGCACTTTCCGAGAT G GATGGGTCTCCTACTATAACCAGC	14220	House mouse
AGAACCCTTCCGCACTTTTCGAGAT G GATGGGTCTCCTACTATAACCAGC	14757	Rat



GENERAL SUMMARY

Summary of results and general conclusion

In the current study, I investigated the effect of genetic factor, single nucleotide polymorphisms (SNPs), on the iron content of beef longissimus dorsi (LD) muscle.

The dual role of iron, essential but toxic at high concentration, requires cellular and systemic regulation of iron homeostasis. Muscle iron content is affected by physiological and environmental factors including age (Giuffrida-Mendoza et al., 2007), muscle type (Doornenbal, 1981), and diet (Miltenburg et al., 1992), and it is also affected by genetic factors. Several human cohort studies have shown that the serum iron status was significantly associated with polymorphisms in iron-related genes (Constantine et al., 2009; Milet et al., 2007), (Benyamin et al., 2009a; Benyamin et al., 2009b). In this study, I focused on one candidate gene, *ferroportin* (*Fpn*), which encodes the only known cellular iron exporter. Nine novel SNPs, NC007300: g.1780 A>G, g.1872 A>G, g.7169 C>T, g.7477 C>G, g.19208 C>T, g.19263 A>G, g.19427 A>G, g.19569 C>T, and g.20480 C>T, were identified in the exons and their flanking regions of *Fpn*. Except g.19427 A>G and g.20480 C>T, all of the SNPs identified were located in intronic region. Nucleotide sequence alignment using BLAST showed that SNP NC007300: g.7169 C>T, g.19208 C>T, g.19263 A>G, and g.20480 C>T, were conserved among cattle, human, chimpanzee, monkey, house mouse, and rat (<http://www.ncbi.nlm.nih.gov/blast>).

Genotypes of the 1086 Angus cattle were obtained for the 9 novel SNPs, and effect of the 9 identified SNPs on muscle iron content was analyzed. SNP g.19208 C>T,

g.19263 A>G, and g.20480 C>T were found to be significantly associated ($P < 0.007$) with the iron concentration in bovine LD muscle. It is noticeable that the three SNPs were conserved among species and SNP g.19208 C>T and g.19263 A>G were located -81 and -26 nt upstream of exon 7, respectively. To preliminarily investigate the mechanism of the strong association observed between iron content and SNP g.19208 C>T and g.19263 A>G, I used an on-line alternative splice site predictor (Wang and Marin, 2006) and found SNP g.19263 A>G to be a few nucleotides away from a putative RNA splicing site. In addition, this polymorphism was not in Hardy-Weinberg equilibrium, which potentially indicates selection at this site. We also found that SNP g.19208 C>T was in a sequence pattern that was reported to be a *cis*-acting element (Shibata et al., 1996). The observation indicated that SNP g.19208 C>T and g.19263 A>G could potentially regulate the efficiency of intron excision, and consequently influence muscle iron content. It is also reported that exonic splicing enhancers (ESEs) are *cis*-acting RNA sequence elements located within exons that can increase exon inclusion by serving as binding sites for the assembly of multicomponent splicing enhancer complexes (Black, 2003). With online bioinformatics tool—ESE Finder (Cartegni et al., 2003; Smith et al., 2006), SNP g.20480 CC genotype was predicted to be in a putative alternative splicing factor/splicing factor 2 (ASF/SF2) binding site (GAGACGG), which was lost if the genotype is changed from C to T. Therefore, it is possible that SNP g.20480 C>T, though a synonymous mutation, would influence the function of Fpn via the interference of the splicing efficiency of exon 8 as a part of ESE.

In addition, polymorphism g. 19208 C>T, g.19263 A>G, and g.20480 C>T were in

nearly complete linkage disequilibrium, which defined two haplotypes, TGC and CAT.

Association analysis for this haplotype block showed that beef from individuals that were homozygous for the TGC haplotype had significantly ($P < 0.001$) higher iron contents than did beef from CAT homozygous or heterozygous individuals.

In conclusion, results of the current study indicated that SNPs, NC007300: g.19208 C>T, g.19263 A>G, and g.20480 C>T in *Fpn* might be useful markers for facilitating the selection of Angus cattle with high LD muscle iron content and therefore to improve the healthfulness of beef.

Future work

1. The mechanism of the SNP effects

In this study, we found strong associations between three SNPs identified and muscle iron content. Two of them were in introns and one in an exon without causing nucleotide replacement. To explain the observed association, we preliminarily analyzed whether they are conserved by sequence alignment among species and analyzed their possible interference with RNA splicing using the bioinformatics tools available online. However, these tools are limited because they performed the function based on available information for mRNA and genomic DNA sequence alignments, whereas biological mechanisms may vary among species, cell types, and even different development stages (ElSharawy et al., 2009). Therefore, systematic and hypothesis-driven *in vivo* and *in vitro* experiments are needed to investigate the effect of the putatively splicing-relevant SNPs. The elucidation of the mechanism of how the SNPs affect muscle iron content would be a

great improvement to the understanding of the molecular basis of Fpn function and iron variation and enrich our knowledge of RNA splicing mechanisms.

2. SNPs in other iron regulation proteins in beef

Skeletal muscle is of great importance regarding whole body iron homeostasis. It is estimated that, in human beings, skeletal muscle represents about 40% of body mass and contains 10% to 15% body iron that is mainly located in myoglobin (Robach et al., 2009). However, little attention has been given to the molecular physiology of iron homeostasis in skeletal muscle. The regulation of iron homeostasis could be different in muscle than in other tissues when considering the high amount of myoglobins and their unique function. Bovine muscle may be used as a model to understand the regulation of iron utilization and storage in muscle. In addition, several gene polymorphisms that are linked with serum iron status have been found in iron homeostasis-related genes, such as *BMP2* (Milet et al., 2007), *CYBRD1* (Constantine et al., 2009), *TF* (Benyamin et al., 2009b), and *TMPRSS6* (Benyamin et al., 2009a). Investigations are needed for the associations between gene polymorphisms in iron-related proteins and the muscle iron content.

Moreover, beef is a good source of dietary iron in regard to both amount and bioavailability. A 100 g portion of raw beef would provide an adult with more than 38.2% of daily iron requirement. Therefore, it would be of great interest to improve the healthfulness of beef from the aspect of iron contents. Identification of SNPs in other iron-related proteins and investigations of their effect on muscle iron content will contribute to our understanding of muscle iron regulation and also will be useful for developing marker-assisted selection of cattle that can produce progeny with more

desirable muscle iron content.

3. The association between SNPs and other minerals

Besides that of iron, the content of many other essential elements are known to vary in beef (Doyle, 1980). Mechanisms of some mineral-loading disorders indicated genetic influence, including disorders for zinc, magnesium, and copper. It is of great interest to know the effect of SNPs in the content of minerals other than iron. Mineral content can be measured in bovine muscle and SNPs can be identified on the genes related to mineral homeostasis. Association analysis can be carried out with SAS.

4. Breed effect on LD muscle iron content

In this study, Angus cattle were used for the identification of SNPs. However, it will be beneficial if the SNPs identified in Angus cattle can be applied also to other breeds of beef cattle. Though previous study showed little breed effect on iron content of muscle (Doornenbal, 1981), future systematic studies are needed to compare the LD muscle iron content among more breeds of cattle in larger population.

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