PRODUCTION OF CHICKEN FOLLISTATIN 315 AND CHICKEN FOLLISTATIN 315 FUSED TO A CHICKEN IgY Fc FRAGMENT IN ESCHERICHIA COLI

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ABSTRACT

Follistatin (Fst) is an autocrine glycoprotein that binds to multiple members of the transforming growth factor family to regulate various physiological processes. Fst has shown to bind myostatin, a potent negative regulator of skeletal muscle growth. Inhibition of myostatin activity by Fst treatment enhanced muscle growth, as well as ameliorates symptoms of muscular dystrophy in animal models, illustrating the potential of Fst as a therapeutic agent to improve muscle growth in animal agriculture or to treat muscle wasting conditions or disease in humans. Therefore, we designed a study to produce biologically active recombinant chicken FST315 (MBP-chFST315) and a chFST315-Chicken IgY constant domain (3-4) fusion protein (MBP-chFST315-Fc(3-4)) in an Escherichia coli host. Complex folding patterns along with multiple disulfide bonds of Fst had made it a difficult challenge to express bioactive Fst protein in E. coli. In this study, we compared combination of vector systems and E. coli strains in order to produce a soluble form of chFST315. Disulfide bond formation of chFST315 is necessary for correct protein folding. Therefore, we expressed the chFST315 protein in either a system that utilizes a periplasmic expression strategy or a genetically modified E. coli system that is capable of disulfide bond formation in the cytoplasm. Periplasmic expression of MBP-chFST315 using the pMAL-p5x vector system failed to produce a soluble recombinant protein. However, when a cytoplasmic expression vector pMAL-c5x was combined with SHuffle® E. coli strain, we were successful in producing a soluble form of MBP-chFST315 and MBP-chFST315-Fc(3-4). The MBP-chFST315 was able to bind to myostatin and to a lesser extent to activin in an in vitro binding assay. MBP-chFST315 inhibited myostatin activity in an in vitro gene reporter assay. The recombinant MBP-
chFST315 will be useful in investigating the potential of Fst in improving skeletal muscle growth of chicken as well as in future studies of investigating signaling pathways involved in skeletal muscle hypertrophy.
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Chapter 1

Literature Review

1.1. Transforming growth factor beta (TGF-β) family proteins and their signaling pathway

The TGF-β family of proteins regulate multiple cellular events during animal development and in adult cell homeostasis (Attisano and Wrana, 2002). TGF-β family of proteins can be further divided into multiple sub-families based on their receptor interactions, and these includes TGF-β, activin, and bone morphogenetic protein (BMP)/growth differentiation factor (GDF) (Innis et al., 2000; Attisano and Wrana, 2002). Table 1 summarizes the list of proteins in these subfamily members. TGF-β, activin, and BMP/GDF subfamilies signal through a receptor complex, which includes type-I and type-II transmembrane serine/threonine kinase receptors (Franzén et al., 1993; Attisano, 2002). Type-I receptors includes Alk4 or Alk5, whereas type-II receptors includes TGF-type II receptor (TGFβRII), activin type II receptor A (ActRIIA), and activin type II receptor B (ActRIIB). TGF-β subfamily of proteins bind to TGFβRII/Alk5 receptors, whereas activin and BMP bind exclusively to ActRIIB/Alk4 or ActIIA/Alk 4 (Fig. 1A) (Franzén et al., 1993; Cárcamo et al., 1994; Byerley et al., 2010).

The three-dimensional shape of TGF-β is commonly referred to “hand-shape motif” (Fig. 2A) (Cash et al., 2009). Both myostatin (MSTN) and activin dimers displays a similar three-dimensional motif to that of other TGF-β proteins (Cash et al., 2009). The hand-shape motif includes four beta strands or “fingers”, a cysteine knot or “palm”, and a
major helix or “wrist” region (Cash et al., 2009). TGF-β monomers are placed in an anti-parallel direction and linked by a disulfide bond in the palm region (Cash et al., 2009). Receptor binding sites of both MSTN and activin dimers are similarly located (Fig. 2B) (Cash et al., 2009). Both contain two distinct convex and two concave surfaces (Cash et al., 2009). The convex surface located near the finger region of TGF-β contains type II receptor binding site, while the concave portion located in the finger/wrist region contains type I receptor binding site (Cash et al., 2009).

The phosphorylation of type I receptors causes a signaling cascade, which involves a family of SMAD transcription factors (Fig. 1B) (Attisano, 2002). After ligand binding, the receptor-regulated R-SMAD (SMAD 1, 2, 3, 5, 8) is phosphorylated at two serine residues by the type I receptor. Phosphorylation of R-SMAD causes the release of R-SMAD from the receptor complex. The R-SMAD then binds to a co-SMAD molecule (SMAD4), leading to nuclear localization of the R-SMAD/co-SMAD complex. Another class of inhibitory SMAD molecules (SMAD6, and 7) negatively regulates the activity of R-SMADS (Attisano, 2002).

Unlike activin and other members of the TGF-β family of proteins, MSTN has been shown to bind to ActRIIB/ActRIIA and Alk4/Alk5 receptors (Fig. 1A) (Rebbapragada et al., 2003). Further analysis of the crystal structure of MSTN revealed that the N-terminal region before the wrist, commonly referred as the prehelix loop is responsible for MSTN ability to utilize both Alk4 and Alk5 type I receptors (Cash et al., 2009). Activin is not able to utilize Alk5 receptors, but replacement of the prehelix loop of activin (residue 45-58) with the prehelix loop of MSTN (residues 48-56) gave the recombinant activin molecule the ability to utilize both Alk4 and Alk5 receptors (Cash et al., 2009).
al., 2009). Binding of MSTN to the receptor complex activates the SMAD signaling cascade, which involves R-SMAD molecules (SMAD2/3) and co-SMAD molecule (SMAD4) (Figure 1B) (Lee and McPherron, 2001; Zhu et al., 2004). Interestingly, MSTN also up-regulates the expression of SMAD7 through a negative feedback mechanism (Zhu et al., 2004).

1.2. Myostatin (MSTN)

Myostatin (MSTN), also called GDF-8, is a strong negative regulator of skeletal muscle growth and development (McPherron et al., 1997). During embryogenesis, MSTN is only expressed in the myotome compartment, a structure which gives rise to skeletal muscle precursor cells (McPherron et al., 1997). MSTN also plays a significant role in skeletal muscle homeostasis of adult animals (McPherron et al., 1997). Adult mammalian species express MSTN predominantly in skeletal muscles in the body (McPherron et al., 1997).

MSTN protein is initially expressed as a latent precursor protein, a characteristic common to the TGF-β family proteins (McPherron et al., 1997). The 376 amino acid sequence of a single MSTN molecule in mice contains an N-terminus signal sequence, prodomain region (267 amino acids, 38 kDa) and a C-terminus active domain with 109 amino acids (12.5 kDa) (McPherron et al., 1997). The C-terminus of MSTN molecule contains nine cysteine residues, and one of which participates in the formation of intermolecular disulfide bond with another MSTN molecule to form a mature MSTN dimer (25 kDa) (McPherron et al., 1997).
Two proteolytic cleavage events occur before the MSTN-prodomain complex is secreted to the extracellular matrix of skeletal muscle cells (McPherron et al., 1997; Thies et al., 2001; Lee, 2008). These events involve removal of the 24-amino acid signal-peptide and cleavage at a specific RSRR (amino acid 263-266) site located between the N-terminal propeptide (prodomain) and C-terminal MSTN (Lee and McPherron, 2001; Huang et al., 2007; Huang et al., 2011). The prodomain region that undergoes cleavage during prepro MSTN processing makes a non-covalent complex formation with MSTN, and suppresses MSTN activity (McPherron et al., 1997; Thies et al., 2001; Anderson et al., 2008). Therefore, removal of prodomain from MSTN dimer is necessary for MSTN biological activity (McPherron et al., 1997; Lee and McPherron, 2001; Wolfman et al., 2003). Removal of the non-covalently bonded prodomain is thought to be done by a member of the bone morphogenetic protein-1/tolloid (BMP-1/TLD) family of metalloproteinases (Wolfman et al., 2003).

A double muscling phenotype is seen in both naturally-occurring, nonfunctional mutation of MSTN and experimental deletion of MSTN (Grobet et al., 1997; Kambadur et al., 1997; McPherron et al., 1997; Schuelke et al., 2004; Clop et al., 2006; Mosher et al., 2007). For example, mice with MSTN gene knocked-out (MSTN^−/−) have nearly 2-3 times greater skeletal muscle mass than wild type mice, and the increase in muscle mass is contributed from both muscle cell hypertrophy and hyperplasia (McPherron et al., 1997). Heterozygous deletion of MSTN (MSTN^+/−) has an intermediate phenotype between the wild type (MSTN^+/+) and homozygous deletion (MSTN^−/−) (McPherron et al., 1997; Szabo et al., 1998; Smith et al., 2000; Schuelke et al., 2004; Mosher et al., 2007).
In contrast, a significant decrease in skeletal muscle mass was observed in transgenic MSTN-overexpressing mice (Reisz-Porszasz et al., 2003).

Double muscling phenotype occurs naturally at a high frequency in Belgian Blue and Piedmontese cattle breeds (Grobet et al., 1997; Kambadur et al., 1997). Both cattle breeds have an autosomal recessive muscular hypertrophy (mh) loci located on chromosome 2, which is also the location of the MSTN loci (Charlier et al., 1995; Grobet et al., 1997). Initial studies were not able to distinguish the expression levels of MSTN between wild type and Belgian Blue and Piedmontese cattle (Kambadur et al., 1997). Unlike the MSTN knockout mice seen before, Belgian Blue and Piedmontese cattle are able to express the MSTN protein (Kambadur et al., 1997). A subsequent study with the Belgian Blue cattle has shown that MSTN is expressed in a truncated and biologically inactive form (Kambadur et al., 1997). This is due to a frame shift mutation in the MSTN gene, where a 11 nucleotide sequence is deleted (Kambadur et al., 1997). In Piedmontese cattle, the non-functional MSTN protein is due to a DNA mutation (G-A) at position 941 of the coding region of the MSTN gene (Kambadur et al., 1997). This mutation replaces a cysteine (amino acid 314) molecule that is highly conserved throughout the TGF-β families to a tyrosine (Kambadur et al., 1997). Hypermuscular phenotype seen in MSTN-deleted species, and decreased muscle mass observed in MSTN over-expressing mice clearly indicate that MSTN negatively regulates the growth and development of skeletal muscles.

1.3. Follistatin
Follistatin (Fst) is an autocrine, secretory glycoprotein that plays a prominent role in mammalian prenatal and postnatal development. Two Fst proteins with molecular weights of 32 and 35 kDa were first discovered in 1987 (Ueno et al., 1987). Initial studies demonstrated that the secretion of follicular stimulating hormone is attenuated by Fst (Bilezikjian et al., 1998). Therefore, it was thought that the biological activity of Fst was restricted to the reproductive system. However, further investigation revealed that Fst is a TGF-β binding protein, and that the biological activity of Fst encompasses multiple organ systems, including bone, skeletal muscle, and liver. There are more than 30 known proteins belonging to the transforming growth factor beta (TGF-β) family of proteins (Table 1) (Lin et al., 2003). Of the multiple TGF-β proteins, the most well-known binding partners of Fst are MSTN, activin and bone morphogenetic proteins (BMPs).

1.3.1. Fst gene expression during myogenesis

Transcriptional regulator proteins augment gene expression by binding to specific regions of DNA such as the promoter, enhancer and silencer regions. The binding of transcriptional regulatory molecules can either promote or silence the expression of a certain gene. Thus, identifying specific transcriptional regulator proteins that bind to distal and proximal promoter regions of Fst can help to explain which mechanisms promote and/or inhibit Fst mRNA expression.

To investigate the mechanism(s) behind Fst gene expression, Egbert de Groot analyzed the DNA coding region upstream of the Fst gene in mice (de Groot et al., 2000). Investigation of the first 500 base pairs upstream of the Fst start codon revealed at least three transcription initiation sites followed by a TATA box sequence (de Groot et al.,
Furthermore, transcriptional regulator binding sites, including AP-1, CREB, SP-1, NFAT, MyoD, Branchyury-T and Tcf, have been identified (de Groot et al., 2000). The presence of these transcription factors are consistent with subsequent studies which demonstrate the involvement of CREB, MyoD and NFAT in Fst mRNA expression during myogenesis (Iezzi et al., 2004; Pisconti et al., 2006; Sun et al., 2010). Furthermore, the activity of Fst promoter is reduced when the expression of MyoD, NFAT, or CREB is inhibited (Pisconti et al., 2006).

According to a study, nitric oxide (NO) is responsible for Fst induction during muscle development (Pisconti et al., 2006), and the induction appears to involve the regulation of many of the above transcription factors (Pisconti et al., 2006). Muscle cell maturation is divided into two distinct stages (Jansen and Pavlath, 2008). Specific genes are expressed for each stage of muscle development (Jansen and Pavlath, 2008). The first stage involves the formation of nascent myoblasts and myotubes (Jansen and Pavlath, 2008). The second stage involves the fusion of the nascent myoblasts and myotubes, forming mature skeletal muscle cells (Jansen and Pavlath, 2008).

Results by Pisconti et al. indicate that this secondary muscle fusion factor is Fst (Pisconti et al., 2006). The NO/cyclic–guanosine monophosphate (cGMP) pathway has been shown to promote Fst expression by recruiting transcription factors, such as NFAT, MyoD, and CREB, to the promoter region of Fst. In the same study, skeletal muscle precursor cells named satellite cells of developing mice were treated with a NO donor (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl) amino] diazen-1-iium-1,2-diolate] (DETA-NO) or a NO synthase inhibitor, N-nitro-Lw-Arginine methyl ester (L-NAME), in order to examine the role of NO in skeletal muscle fusion during muscle development (Pisconti
et al., 2006). Myoblast fusion and the amount of bi-nucleated/multi-nucleated myotubes were increased by the treatment of DETA-NO (Pisconti et al., 2006). The treatment of L-NAME, however, decreased the fusion index (Pisconti et al., 2006). Injections of DETA-NO and L-NAME at different time frames during development have shown that the effect of NO on muscle fusion peaks only at early stages of development (Pisconti et al., 2006). The treatment of DETA-NO and L-NAME at later stages of development had no effect on either fusion index or the number of multi-nucleated cells. The results together suggest that NO signaling may be time-dependent and is expressed only during the secondary cell fusion (Pisconti et al., 2006).

The effect of NO in satellite cell fusion is believed to be due to the production of cyclic guanosine monophosphate (cGMP) that is produced by guanylate cyclase (Pisconti et al., 2006). The treatment of cGMP inhibitor 1H-(1, 2, 4) oxadiazolo [4, 3-oa]quinoxalin-I-one (ODQ) reversed the increased fusion index observed in DETA-NO treated satellite cells. A cGMP analog (Br-cGMP), however, emulated the increased fusion index observed previously in DETA-NO treated satellite cells. The decreased fusion index observed in ODQ treated satellite cells were reversed with the addition of Br-cGMP, suggesting that the production of cGMP is necessary for satellite cell fusion (Pisconti et al., 2006).

Fst mRNA has been isolated in multiple tissues, suggesting that the physiological role of Fst is not solely involved in muscle homeostasis (Phillips and de Kretser, 1998). For example, in ovarian development, the expression of Fst mRNA has been found to be up-regulated by BMP2 and forkhead-domain transcription factor L2, and down-regulated
by GDF9 (Kashimada et al., 2011; Shi et al., 2011). This suggests that the mechanism involved in Fst gene expression may alter from one tissue type from another.

1.3.2. Epigenetic regulation of Fst mRNA expression

Recent studies have suggested that Fst mRNA expression is governed by a complex epigenetic mechanism (Utriainen et al., 2006; Sun et al., 2010). Both DNA methylation and DNA deacetylation have been found to restrict DNA transcription. DNA methylation at specific methyl CpG-binding domain causes further coiling of DNA which restricts the binding of transcription factors (Wade, 2001). An estimated 8% of all nucleotides found in the Fst promoter region is susceptible to DNA methylation (Utriainen et al., 2006). In a study, Fst mRNA expression was quantified by real time PCR in NCI-H295R human adrenocortical cells following treatment with a DNA methylation inhibitor 5-Aza-2-deoxycytidine (Azad) (Utriainen et al., 2006). Inhibition of DNA methylation caused an 2-5 fold increase in both Fst288 and Fst315 mRNA expression in a dose dependent manner (Utriainen et al., 2006). This suggest that DNA methylation of the Fst promoter regulates the expression of Fst mRNA (Utriainen et al., 2006).

Similar to DNA methylation, deacetylation of DNA by histone deacetylases (HDAC) also inhibits DNA transcription by restricting the ability of transcription factors to bind to regulatory binding sites. Studies have shown that HDAC negatively regulate Fst mRNA expression (Iezzi et al., 2002). In contrast, the treatment of HDAC inhibitor trichostatin A (TSA) on undifferentiated skeletal myoblasts increased the expression of Fst mRNA (Iezzi et al., 2002). This suggests that deacetylation of the Fst promoter by a
HDAC molecule may negatively regulate the expression of Fst (Iezzi et al., 2002). In a subsequent study, Iezzi et al. (2004) further investigated the effects of HDAC inhibitor on myoblast fusion through induction of Fst (Iezzi et al., 2004). This study demonstrated that the treatment of TSA promoted the recruitment of transcription activators, such as CREB and NFAT, to the promoter site of Fst in undifferentiated skeletal myoblasts (Iezzi et al., 2004).

Further studies suggest that HDAC4, a skeletal muscle specific histone deacetylase molecule, is capable of inhibiting Fst expression by a process which involves deacetylation of the Fst promoter (Sun et al., 2010). Furthermore, the expression of Fst and HDAC4 inhibitor molecule micro RNA one (miRNA-1) is thought to be mediated by the mammalian target of rapamycin (mTOR) signaling pathway (Sun et al., 2010). Interestingly, a previous study demonstrated the ability of MyoD to bind to miR-1 enhancer region (Zhao, 2005). The study conducted by Sun et al., confirmed that the expression of miRNA-1 is enhanced by the binding of MyoD to the promoter region of miR-1 (Sun et al., 2010). Inhibition of the mTOR pathway by rapamycin, in contrast, diminished the expression of miRNA-1 and MyoD, suggesting that both MyoD and miRNA-1 expression are downstream targets of the mTORC1 signaling pathway (Sun et al., 2010). Injection of TSA into the transverses abdominis muscles in mice has shown to increase the activity of mTORC1, MyoD, and miRNA-1 (Sun et al., 2010). This, in turn, allowed the Fst promoter region to be hyper-acetylated (Sun et al., 2010).

1.3.3. Multiple Fst isoforms
The Fst gene is highly conserved among mammalian species. For example, there is a 95% similarity in amino acid sequence between chicken and rat Fst (Connolly et al., 1995). The 6 kb Fst gene contains 6 exons and 5 introns (Fig. 3A) (Shimasaki et al., 1988). During transcription of Fst DNA into mRNA, the Fst mRNA undergoes alternative splicing to form two precursor isoforms of Fst with amino acid sequences of either 344 (Fst344) or 317 (Fst317) (Ueno et al., 1987; Shimasaki et al., 1988).

Alternative splicing occurs between the 3’ end of exon 5 (Shimasaki et al., 1988). Cleavage of intron 5 and exon 6 generates a stop codon (TGA) at the end of exon 5, leading to the formation of the carboxyl-truncated Fst precursor Fst317 (Lin et al., 2008). Splicing of all 5 introns of the 6 kb Fst gene does not produce an in-frame stop codon at the 3’ end of exon 6 (Lin et al., 2008). Exon 6 is believed to be separated into two regions and is referred to as exon 6a and exon 6b (Lin et al., 2008). The in-frame stop codon (TAA) at the end of exon 6b is generated when intron 5 and exon 6a is spliced together (Lin et al., 2008). This process produces the Fst344 precursor molecule (Lin et al., 2008). Exon 1 of both Fst344 and Fst317 codes for a signal peptide (29 amino acids), thus cleavage of the signal sequence produces the mature forms of Fst of 315 (Fst315) and 288 (Fst288), respectively (Fig. 3B) (Lin et al., 2008).

Isolation of Fst from porcine ovaries revealed an additional isoform of Fst of 303 amino acids (Fst303) (Sugino et al., 1993). Fst315 and Fst303 share similar characteristics of having a highly acidic carboxyl-terminal end (Glu^{292}-Asp-Thr-Glu-Glu-Glu-Glu-Glu-Glu-Asp-Glu-Glu-{\text{Asp}}^{302}) (Sugino et al., 1993; Lerch et al., 2007). Since Fst288 lacks the acidic carboxyl-terminal end, it is believed that Fst303 is formed by a post-translational proteolytic cleavage of Fst315 (Sugino et al., 1993). The mechanism
underlying the proteolysis of Fst315 is not well known. It is thought that the acidic carboxyl terminal domain may promote certain carboxypeptidase to cleave the Fst315 protein up to the Gln\textsuperscript{303} residue (Sugino et al., 1993).

The three main isoforms of Fst are Fst315, Fst288, and Fst303. However, when considering the state of Fst glycosylation, further derivatives of Fst are possible (Sugino et al., 1993). Amino acid analysis of Fst shows two possible sites of glycosylation at Asn\textsuperscript{95} and Asn\textsuperscript{259} (Sugino et al., 1993; Hyuga et al., 2004). Therefore, four potential isoforms of Fst are possible for each main Fst molecule, one fully-glycosylated molecule, two single-glycosylated molecules and one non-glycosylated molecule (Sugino et al., 1993; Hyuga et al., 2004). In actuality, only a small fraction of the possible Fst isoforms has been identified (Sugino et al., 1993). For example, only six molecules of Fst were isolated from porcine ovary, including one Fst315 molecule glycosylated at Asn\textsuperscript{259}, three Fst303 molecule glycosylated at both Asn\textsuperscript{95}-Asn\textsuperscript{259}, Asn\textsuperscript{259} only, a non-glycosylated isoform, and two Fst288 molecule glycosylated at Asn\textsuperscript{259} only, and a non-glycosylated Fst288 isoform (Sugino et al., 1993).

1.3.4. Disulfide bond formation of Fst

An important characteristic of Fst is the presence of multiple intramolecular disulfide bonds. Both Fst315 and Fst288 protein contains 36 cysteine residues, thus formation of 18 disulfide bonds is possible (Sidis et al., 2001). Disulfide bond formation in Fst do not occur between adjacent cysteine residues, and disulfide bond formation only occurs between cysteine residues of the same domain (Fig. 4) (Hohenester et al., 1997). Sidis's experiment on disulfide bond disruption demonstrated that replacement of two
cysteine residues at positions 26 and 27 with alanine, reduced activin binding affinity to less than 5% of the wild type (Sidis et al., 2001). The removal of only two disulfide bonds of Fst completely disrupted Fst capability to bind to its ligand, suggesting that disulfide bond formation is critical to the biological activity of Fst (Sidis et al., 2001).

1.3.5. Fst protein domains

Fst can be divided into four domains, consisting of a signal sequence, an amino-terminus domain (N-terminal), three Fst domains and a carboxyl-terminus domain (C-terminal), as illustrated in Fig. 3A and B (Shimasaki et al., 1988). The signal sequence consist of 27-29 amino acid residues depending on animal species (Connolly et al., 1995). However, because the signal sequence is cleaved during protein translation, the final product results in an Fst protein of the same length. The function of the signal sequence is to facilitate the translation of Fst mRNA in the rough endoplasmic reticulum (Kumar, 2005; Saito et al., 2005).

The 63-residue N-terminal domain of Fst is believed to have a significant importance in Fst biological activity (Sidis et al., 2001). Deletion of the N-terminal domain or truncation of the first two residues (Gly\(^1\)-Asn\(^2\)) of Fst288, reduced the activin binding potency to 5% of the wild type (Sidis et al., 2001). This suggests that the three Fst domains alone do not have the ability to inhibit the biological activity of activin (Sidis et al., 2001). However, it is important to note that the N-terminal domain alone is not sufficient for Fst to bind to activin (Keutmann et al., 2004). Multiple experiments demonstrated that Fst domains 1, 2 and the N-terminal domain are the minimum requirement in order for Fst288 to bind to activin (Sidis et al., 2001; Keutmann et al.,
Deletion of hydrophobic amino acids in the N-terminal domain has also shown to disrupt activin binding (Sidis et al., 2001). Hydrophobic amino acid deletion resulted in reduced binding affinity of up to 19% of the wild type phenotype (Sidis et al., 2001).

The domains following the N-terminal domain are three similar Fst domains (FstD1, FstD2, and FstD3) coded by exons 3-5 (Shimasaki et al., 1988; Keutmann et al., 2004). Each FstD (1-3) contains about 63-65 non-cysteine amino acids and 10 cysteine amino acids (Sidis et al., 2001). Much like the N-terminal domain, FstD1 and FstD2 are shown to be necessary for Fst activity (Keutmann et al., 2004). Deletion of FstD1 and/or FstD2 diminished activin binding affinity to less than 5% of the wild type (Keutmann et al., 2004). However, deletion of FstD3 retained 60% binding affinity as compared with the wild type, demonstrating that deletion of FstD3 is tolerable (Keutmann et al., 2004). The order in which the FstD(1-3) is formed has also shown significant importance in biological activity (Keutmann et al., 2004). Activin binding capability was significantly reduced by either rearranging the FstD (FstD-2/1/3 and FstD-3/1/2) or doubling one FstD (FstD-1/1/3 and FstD-2/2/3) (Keutmann et al., 2004).

The C-terminal, as mentioned before, is removed from Fst288 by alternative splicing of the original Fst mRNA (Shimasaki et al., 1988). The 27 amino acid sequence of the C-terminal contains a chain of ten acidic amino acids (Glu$^{292}$-Asp$^{293}$-Thr$^{294}$-Glu$^{295}$-Glu$^{296}$-Glu$^{297}$-Glu$^{298}$-Glu$^{299}$-Asp$^{300}$-Glu$^{301}$-Asp$^{302}$) commonly referred to as the acidic tail of Fst315 (Sugino et al., 1993; Lerch et al., 2007). Structural analysis of Fst315 demonstrated that the acidic tail resides in the groove formed by FstD2 and FstD3, suggesting that FstD2 and FstD3 may guide the proper folding of Fst315 (Lerch et al., 2007). Closer investigation of the interaction between the acidic tail and the FstD2-FstD3
groove revealed that basic residues (Arg$^{140}$, Arg$^{200}$, and Arg$^{237}$) found in the groove may potentially form hydrogen-bonds with acidic amino acids of the tail (Glu$^{280}$, Glu$^{296}$, and Glu$^{297}$) (Lerch et al., 2007). The C-terminal is believed to diversify the biological activity of Fst by altering the binding characteristics to different ligand molecules (Sugino et al., 1993).

1.3.6. Fst as a TGF-β binding protein

Fst was originally found to be an activin binding protein, but further studies suggest that Fst may also bind to other members of the TGF-β family of proteins, including MSTN and BMP-4 (Nakamura et al., 1990; Fainsod et al., 1997; Lee and McPherron, 2001). Until recently, the ligand binding characteristic of Fst was not well understood. Analysis of the crystal structure of Fst315-activin complex revealed that two Fst315 molecules bind to one activin dimer (Lerch et al., 2007). A number of subsequent studies support two Fst molecules of the same isoform bind to one TGF-β dimer (Cash et al., 2009; Cash et al., 2012). MSTN and activin initiate the SMAD signaling pathway by binding to both type-I and type-II receptors (ActRIIB/Alk4 or ActIIA/Alk 4, Fig. 1A) (Franzén et al., 1993; Cárcamo et al., 1994; Byerley et al., 2010). The MSTN dimer contains two concave- and two convex-like shapes (Fig. 2B) (Lerch et al., 2007; Cash et al., 2009). The concave and convex portions of MSTN are the locations of type-I and type-II receptor recognition site, respectively (Lerch et al., 2007; Cash et al., 2009). Fst inhibits MSTN and activin by covering both convex region of type II receptor binding site, and concave region of type I receptor binding site, thereby preventing the abilities of MSTN and activin to bind to their respective receptors (Lerch et al., 2007; Cash et al., 2009).
Although most Fst isoforms are capable of binding to MSTN and activin, the binding affinities differ between each specific ligand and the three major isoforms of Fst (Nakatani et al., 2008). The binding affinity ($K_D$) for Fst288 to activin has been reported to be 1.72 nM (Hashimoto et al., 2000; Nakatani et al., 2008). However, the binding affinity for Fst315 to activin has been reported to be 10 times lower than Fst288, and Fst303 had an intermediate binding affinity (Sugino et al., 1993; Cash et al., 2009). Fst binding affinity to MSTN has been reported to be lower than activin (12.3 nM) (Nakatani et al., 2008). Furthermore, the association constant for Fst288 binding to activin has been reported to be 15 fold higher than MSTN (Cash et al., 2009). Unlike their binding to activin, Fst315 and Fst288 isoforms bind to MSTN with similar affinities (Cash et al., 2009).

### 1.3.7. Degradation of Fst-Ligand complex

In addition to blocking receptor binding, Fst may inhibit the activity of MSTN and activin by aiding in the degradation of the ligand molecules through the enhancement of the binding affinity of ligand to cell surface heparin sulfate and subsequent endocytosis (Cash et al., 2009). Cell surface heparin sulfate proteoglycans undergo endocytosis as a major part of metabolic turnover (Yanagishita and Hascall, 1992). Steps involved in the heparin sulfate proteoglycan-induced endocytosis can be separated into four major events, binding of the ligand to heparin sulfate, endocytosis of the ligand, lysosomal degradation, and finally the excretion of the digested ligand back into the environment (Yanagishita and Hascall, 1992). Several key evidences support that the degradation of MSTN and activin is mediated by heparin sulfate proteoglycan-induced endocytosis (Ueno et al., 1987; Hashimoto et al., 1997). Studies have found that the
lysine and arginine-rich amino acid sequence (72-86) located in FstD1 contained the
binding site to heparin sulfate (Ueno et al., 1987; Inouye et al., 1992).

In one study, rat pituitary cell culture was treated with radio-labeled activin and
Fst at varying concentrations (Hashimoto et al., 1997). Activin was radio-labeled using
the chloramine-T method (Greenwood et al., 1963). After treatment with radio labeled-
activin, the cell culture medium was then isolated and treated with trichloroacetic acid, a
chemical commonly used to precipitate large macro molecules (Hashimoto et al., 1997).
After centrifugation, the supernatant was separated from the pellet, thus removing any
undigested radio-labeled activin (Hashimoto et al., 1997). Trichloroacetic acid is unable
to precipitate small peptides, thus if the activin/Fst complex was endocytosed and
subsequently degraded, an increase in radio activity was expected in the supernatant
(Hashimoto et al., 1997). Radio-labeled activin was found in the cell culture medium in
the study, suggesting that activin must have been degraded within the cell and
subsequently removed from the cytoplasm (Hashimoto et al., 1997).

The three main isoforms of Fst have differing binding affinity to cell surface
heparin (Sugino et al., 1993). The presence of the carboxyl domain in both Fst315 and
Fst303 is believed to interfere with the binding affinity to surface heparin sulfate
proteoglycans (Sugino et al., 1997). Fst315 has the lowest affinity to heparin sulfate
followed by Fst303 and Fst288 (Hashimoto et al., 1997). Similar to activin, the acidic tail
of Fst315 and Fst303 is believed to interfere with heparin binding (Sugino et al., 1993;
Hashimoto et al., 1997). Due to a reduced binding affinity for cell surface heparin sulfate
proteoglycans, Fst315 is believed to be the isoform of Fst most abundant in serum, and
Fst288 is believed to be the cell surface-associated isoform (Sugino et al., 1993; Schneyer et al., 2004).

1.3.8. Modulation of muscle mass by manipulation of Fst activity

Substantial evidence supports that Fst is a MSTN binding protein, thus it was postulated that Fst plays a role in skeletal muscle growth and development (Lee and McPherron, 2001). Fst over-expressing transgenic mice have shown to have 197-327% increase in skeletal muscle mass in comparison to wild type mice (Lee and McPherron, 2001). Increased skeletal muscle mass was due to both muscle cell hypertrophy and hyperplasia (Lee and McPherron, 2001). Furthermore, the increase in muscle mass was far greater in Fst transgenic mice than that of both MSTN knockout mice and MSTN-propeptide over-expressing mice, suggesting that Fst is a potent inhibitor of MSTN \textit{in vivo} (Lee and McPherron, 2001). In another study, MSTN null mice were cross breed with an Fst transgenic mice in order to determine if the increase in skeletal muscle mass in the Fst transgenic mice was solely due to the inhibition of MSTN (Lee and McPherron, 2001; Lee, 2007). MSTN null mice carrying the Fst transgene had a far greater increase in skeletal muscle mass, even surpassing the increase seen in both Fst trangenetic mice, and MSTN null mice (Lee, 2007). These findings suggest that Fst may play a role in skeletal muscle growth, which involves a far more complex mechanism than just the inhibition of MSTN (Lee, 2007; Gilson et al., 2009).

In contrast to Fst overexpression, mice with an homozygous deletion of the Fst gene (Fst $^{-/-}$) have been shown to have retarded growth along with respiratory defects, which results in the death of the organism quickly after birth (Matzuk et al., 1995).
Premature ovarian failure was observed in mice with an ovarian tissue specific deletion of Fst, indicating that Fst is necessary for proper development of multiple organ systems (Jorgez et al., 2004).

1.3.9. Fst as a potential therapeutic agent for the treatment of skeletal muscle atrophic disease and conditions

The loss of muscle mass due to skeletal muscle degenerative diseases such as Duchenne muscular dystrophy, or cancer cachexia has serious health consequences. For example, cachexia is characterized as a condition of drastic weight loss and muscle wasting associated with chronic diseases such as cancer and is thought to be responsible for 25-30% of all cancer-related deaths (Tan and Fearon, 2008; Tisdale, 2010). Overexpression of MSTN in mice have shown symptoms of skeletal muscle atrophy, which mimics the symptoms of muscle atrophy and debilitating weakness seen Duchene muscular dystrophy (Reisz-Porszasz et al., 2003). Furthermore, administration of MSTN in mice has also shown to develop cachexia-like symptoms (Zimmers et al., 2002). Other MSTN inhibitory molecules includes Fst (Lee and McPherron, 2001; Kota et al., 2009; Rodino-Klapac et al., 2009), anti-MSTN antibody (Kim et al., 2006), activin type II receptors (Benny Klimek et al., 2010), and MSTN pro-domain (Yang et al., 2001).

Inhibition of MSTN signaling has been demonstrated to be a viable therapeutic strategy in ameliorating muscle wasting (Lee and Glass, 2011). MDX mice originally developed in 1984 has a nonsense mutation in exon 23 of the dystrophin gene, and this animal model mimics the phenotype seen in Duchene muscular dystrophy (Bulfield et al., 1984). Administration of Fst through a gene therapy vector in MDX mice has shown to increase muscle mass as comparable to the wild type mice (Rodino-Klapac et al., 2009).
Furthermore, mice treated with the therapeutic agent also show a dose-dependent increase in grip strength (Rodino-Klapac et al., 2009). In a subsequent study, the same treatment has also been shown to be effective in increasing skeletal muscle mass of a nonhuman primate (Kota et al., 2009). Recent studies have shown that inhibition of the MSTN signaling pathway as a potential therapeutic treatment for cachexia (Benny Klimek et al., 2010; Zhou et al., 2010; Lee and Glass, 2011). Zhou et al. (2010) demonstrated that treating mice with cachexia with an activin type II receptor not only preserved skeletal muscle mass, but also increased the survivability of the mice.

Significant evidence suggests that Fst is a potent inhibitor of MSTN and that Fst may be a potential therapeutic agent to treat muscle wasting conditions (Lee and McPherron, 2001; Rodino-Klapac et al., 2009). Gene therapy and protein treatment of Fst in MDX mice has shown to have immediate and long term beneficial effects of increased muscle strength by inhibiting muscle wasting (Nakatani et al., 2008; Rodino-Klapac et al., 2009). Demonstration of prolonged maintenance of grip strength has important implications for developing a long term treatment to ameliorate symptoms of muscle wasting. However, gene therapy may have limitations for clinical applications due to ethical concerns and unidentified potential side effects. Therefore, new treatments based on the administration of recombinant protein may prove to be an effective and practical approach in the treatment of muscle wasting disease and conditions.
Table 1. Transforming growth factor-beta subfamilies.

<table>
<thead>
<tr>
<th>Sub families</th>
<th>Types</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transforming growth factor beta (TGF- β)</td>
<td>TGF- β- 1, 2, 3</td>
<td>(Innis et al., 2000)</td>
</tr>
<tr>
<td>Bone morphogenetic proteins (BMP) and Growth differentiation factor (GDF)</td>
<td>BMP-2, 4, 5, 6, 7, 9, 10 GDF-5, 6, 7, 8</td>
<td>(Kawabata et al., 1998; Innis et al., 2000; Cheng et al., 2003)</td>
</tr>
<tr>
<td>Activins</td>
<td>Activin AB, Activin A, Activin B</td>
<td>(Innis et al., 2000; Muenster et al., 2011)</td>
</tr>
</tbody>
</table>
Figure 1. (A) Cell signaling receptors of TGF-β, MSTN, and activin (modified from Cash et al., 2009). (B) Signaling pathway of MSTN and activin (modified from Lee and Glass, 2011).
Figure 2. (A) Depiction of MSTN three dimensional structure. (B) Location of TGF-β receptor binding sites (modified from Cash et al., 2009).
Figure 3. (A) Diagram of Fst alternative splicing (modified from Rodino-Klapac et al., 2009). (B) Depiction of Fst288 and Fst315 molecules (modified from Cash et al., 2009).
Figure 4. Predicted location of disulfide bonds in chFst315.
Chapter 2

Production of chicken follistatin315 and chicken follistatin315 fused to a chicken IgY Fc fragment in *Escherichia coli*

ABSTRACT

Follistatin (Fst) is an autocrine glycoprotein that binds to multiple members of the transforming growth factor family to regulate various physiological processes. Fst has been shown to bind myostatin, a potent negative regulator of skeletal muscle growth. Inhibition of myostatin activity by Fst treatment enhanced muscle growth as well as ameliorates symptoms of muscular dystrophy in animal models, illustrating the potential of Fst as a therapeutic agent to improve muscle growth in animal agriculture or to treat muscle wasting conditions or disease in humans. Therefore, we designed a study to produce bioactive recombinant chicken FST315 (MBP-chFST315) and a chFST315-Chicken IgY constant domain (3-4) fusion protein (MBP-chFST315-Fc(3-4)) in an *Escherichia coli* host. Complex folding patterns along with multiple disulfide bonds of Fst had made it a difficult challenge to express bioactive Fst protein in *E. coli*. In this study, we compared combination of vector systems and *E. coli* strains in order to produce a soluble form of chFST315. Disulfide bond formation of chFST315 is necessary for correct protein folding. Therefore, we expressed the chFST315 protein in either a system that utilizes a periplasmic expression strategy, or a genetically modified *E. coli* system that is capable of disulfide bond formation in the cytoplasm. Periplasmic expression of chFST315 using the pMAL-p5x vector system failed to produce a soluble recombinant protein. However, when a cytoplasmic expression vector pMAL-c5x was combined with
SHuffle® E. coli strain, we were successful in producing a soluble form of MBP-chFST315 and MBP-chFST315-Fc(3-4). The MBP-chFST315 was able to bind to myostatin and to a lesser extent to activin in an *in vitro* binding assay. MBP-chFST315 inhibited myostatin in an *in vitro* gene reporter assay. The recombinant MBP-chFST315 produced in this study will be useful in investigating the potential of Fst in improving skeletal muscle growth of chicken as well as in future studies of investigating signaling pathways involved in skeletal muscle hypertrophy.
2.2. Introduction

Myostatin (MSTN), also called GDF-8, is a member of the transforming growth factor β (TGF-β) super family of secreted growth factors and is predominantly expressed in the skeletal muscle (McPherron et al., 1997). A double muscling phenotype is observed in animals with an homozygous deletion of MSTN (Grobet et al., 1997; Kambadur et al., 1997; McPherron et al., 1997; Schuelke et al., 2004; Clop et al., 2006; Mosher et al., 2007). In contrast, overexpression of MSTN in mice have shown symptoms of skeletal muscle atrophy, which mimics the symptoms of debilitating weakness seen Duchene muscular dystrophy (Reisz-Porszasz et al., 2003), suggesting that MSTN is a strong negative regulator of skeletal muscle growth and development.

Inhibition of MSTN by administration or overexpression of anti-MSTN binding proteins, such as follistatin (FST) (Lee and McPherron, 2001; Kota et al., 2009; Rodino-Klapac et al., 2009), anti-MSTN antibody (Kim et al., 2006), activin type II receptors (Benny Klimek et al., 2010), and MSTN pro-domain (Yang et al., 2001) has shown to increase muscle mass, illustrating that inhibition of MSTN may be a viable therapeutic strategy in ameliorating muscle wasting. Transgenic mice overexpressing Fst have shown to have 197-327% increase in skeletal muscle mass in comparison to wild type mice (Lee and McPherron, 2001), suggesting that Fst is a potent inhibitor of MSTN in vivo. Gene therapy and protein treatment of Fst in MDX mice has shown to have immediate and long term beneficial effects of increased muscle strength by inhibiting muscle wasting in mice and nonhuman primate (Nakatani et al., 2008; Kota et al., 2009; Rodino-Klapac et al., 2009).
These studies together illustrate that Fst would be a potential therapeutic agent for the treatment of skeletal muscle atrophic disease and may also have uses in improving skeletal muscle growth in meat-producing animals. In this regard, the ability to produce large quantity of Fst is very important in future investigation of its therapeutic potential. *Escherichia coli* remain a popular choice for recombinant protein production due to its relative low cost and high yields (Chou, 2007). A previous study, however, demonstrated that Fst was expressed as inclusion bodies in *E. coli* (Inouye et al., 1991). The objective of the present study was to examine different vector systems and *E. coli* strains to produce soluble and bioactive Fst315 protein in *E. coli*. 
2.3. Materials and Methods

2.3.1. Construction of chicken follistatin315 and chicken immunoglobulin Y (IgY) constant domain 3-4 DNA

Two pMAL-p5x/c5x vector constructs were created, one containing only chicken follistatin 315 (chFST315) DNA insert, and one containing chFST315 fused to N-terminal chicken immunoglobulin Y constant domain 3-4 (Fc(3-4)) DNA (Fig.5). The fusion of chFST315 and Fc(3-4) insert was mediated by a common SalI restriction enzyme site.

The chFST315 DNA insert (GenBank: X87609.1) was commercially synthesized and subcloned into pUC-57 cloning vector (Genescript, PA). The original chFST315 DNA codon (Appendix 1) was optimized for *E. coli* expression by Genescript before synthesis (Appendix 2). The chFST315 insert contained a 5' SmaI and a 3'SalI restriction enzyme sites. The signal sequence, which is not required in *E. coli* expression, was not added in the insert. Chicken Fc(3-4) DNA sequence was amplified in a polymerase chain reaction (PCR) using chicken spleen cDNA library (Zyagen, CA) as a DNA template (Appendix 8). The sense and antisense primers were based on a report by Parvari et al. (Parvari et al., 1988), and they were 5'-GACGGCGCTCAGAGCTGC-3' and 5'-TTATTTACCAGCCTGTTCCTGCAG-3', respectively. An annealing temperature gradient of 55.6-56.6 °C was applied during PCR amplification. The amplicon was then separated in a 1% agarose gel. The Fc(3-4) fragment was then excised and purified from the agarose gel (Qiagen, CA). 5' SalI and 3' HindIII restriction enzyme sites were added to the Fc(3-4) fragment using PCR method with primer sets containing the restriction enzyme sites, and the purified Fc(3-4) fragment being used as a DNA template. The sense
and antisense primers were 5’-TTTTTGTGACGCGCTTCAGAGCTG-3’ and 5’AAGCTTTATTTACCAGCTTTCTCAGCGTGCG-3’, respectively. Temperature gradient described earlier was used in the PCR. The Fc(3-4) insert containing the restriction enzyme sites was purified from the gel by agarose gel DNA extraction. The predicted DNA sequence is shown in Appendix 4.

2.3.2. Cloning of chFST315 and Fc(3-4) into an expression vector and transformation of expression vectors

To prepare the chFST315-pMAL-p5x/c5x plasmid, 1 µg of chFST315-pUC-57 plasmid was digested with 10 units of SmaI and 10 units of SalI. The excised chFST315 fragment was separated by agarose gel electrophoresis and purified. Also, 1 µg of pMAL-p5X/c5X plasmid (New England Biolabs Inc., MA) was digested with 10 units of XmnI and 10 units of SalI, followed by agarose gel electrophoresis and purification of the digested plasmid from the agarose gel. Following the manufacture’s protocol, the chFST315 DNA fragment was inserted into the pMAL-p5X/c5X vector using a Quick T4 DNA Ligase (New England Biolabs Inc., MA). The chFST315-pMAL-p5X/c5X plasmid was transformed into NEB-Express E. coli (New England Biolabs Inc., MA). Blank pMAL-p5X/c5X was also transformed into NEB-Express E. coli. The newly transformed E. coli was then plated on a Luria broth (LB) agar plate containing ampicillin (100 µg/mL). The following day, a single colony from the transformation plate was then inoculated in 5 mL of LB broth (100 µg/mL ampicillin). After an overnight growth (16 hours, 37°C), the chFST315-pMAL-p5X/c5X plasmid was extracted using a plasmid extraction mini-prep kit (Qiagen, CA) to confirm correct insertion by DNA sequence analysis (Appendix 14) (Operon, CA).
To prepare the chFST315-Fc(3-4)-pMAL-p5x/c5x plasmid, 1µg of chFST315-pMAL-p5X/c5x plasmid was digested with 10 units of SalI. 1 µg of Fc(3-4) fragment was then digested with 10 units of SalI and 10 units of HindIII, and purified after agarose gel fractionation. The Fc(3-4) fragment was then inserted into the chFST315-pMAL-p5x/c5x plasmid as previously described. The resulting construct was then transformed into NEB-Express E. coli. After an overnight growth (16 hours, 37°C), the chFST315-Fc(3-4)-pMAL-p5x/c5x plasmid was extracted using a plasmid extraction mini-prep kit (Qiagen, CA) to confirm correct insertion by DNA sequence analysis (Appendix 15) (Operon, CA).

Shuffle® and NEB-Express E. coli (New England Biolabs Inc., MA) were transformed with either chFST315-pMAL-c5x or chFST315-Fc(3-4)-pMAL-c5x plasmids to induce cytoplasmic expression of recombinant proteins. To induce periplasmic expression, chFST315-pMAL-p5x and chFST315-Fc(3-4)-pMAL-p5x were transformed in only NEB-Express.

2.3.3. Expression of MBP, MBP-chFST315, and MBPchFST315-Fc(3-4) fusion protein

After confirmation of correct insertion, overnight culture of each E. coli cell type was prepared by inoculating 1-10 mL of LB broth containing 100 µg/mL ampicillin with a single colony. The overnight culture was then grown at 30°C for 16 hours. One hundred volumes of new LB broth containing 100 µg/mL ampicillin was then inoculated with the overnight culture. The culture was grown at 30°C. After reaching to an optical density of 0.3-0.4 A (600 nm), 1 mL of total culture was removed (uninduced fraction), and with the
remaining culture, protein expression was induced for 4 hours at 30°C by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 4 mM.

### 2.3.4. Cytoplasmic protein extraction from *E. coli* transformed with the pMAL-c5x expression system

After induction, *E. coli* pellet was harvested by centrifugation at 8,000 g for 10 minutes. The cell pellet was then resuspended with B-PER bacterial extraction reagent (4 mL reagent /1 g of *E.coli* wet weight) (Pierce, Rockford, IL). 2 µL of lysozyme (50 µg/mL) and 2 µL of DNase 1 (2,500 units/mL) was added per 1 mL of B-PER extraction reagent. After an 15 minute incubation at room temperature, the sample was diluted with an affinity column buffer (200 mM NaCl, 20 mM Tris, 1 mM EDTA, pH 7.2) to reach to a final concentration of 10 mL per gram of cell wet weight. The pellet suspension was then sonicated for 2 minutes with 15 second pulses. The soluble crude extract was obtained by centrifugation of the total extract at 8,000 g for 30 minutes. Total, soluble and pellet fraction obtained from 100 µL equivalent of cell culture growth medium was analysed by SDS-PAGE gel electrophoresis.

### 2.3.5. Periplasmic protein extraction from *E.coli* transformed with the pMAL-p5x expression system

Periplasmic fraction was collected following the method described by Chang et al (Chang et al., 2006). Briefly, 100 mL of each culture was centrifuged at 4,000 g for 10 minutes. After removing the supernatant, the pellet was resuspended in 10 mL sucrose buffer (30 mM Tris, 20% sucrose, 1 mM EDTA, pH 8.0). Following a 10 minutes incubation at room temperature, the cells were harvested by centrifugation at 8,000 g for
10 minutes at 4°C. The pellet was then resuspended in 10 mL of 5 mM MgSO$_4$. The osmotic shock fluid containing the periplasmic protein fraction was then obtained by centrifugation at 8,000 g at 4°C for 30 minutes. Total, soluble and pellet fraction obtained from 100 µL equivalent of cell culture growth medium was analysed by SDS-PAGE gel electrophoresis.

2.3.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed with gels containing 12.5%, or 8% polyacrylamide and 0.1% SDS following the procedure by Laemmli (Laemmli, 1970). Samples were mixed with 3X loading buffer which are under either reducing (with 2-mercaptoethanol) or non-reducing (without 2-mercaptoethanol) conditions. Before loading the sample onto the SDS-PAGE gel, samples were boiled at 100°C for 5 minutes. 4 µL of protein standard (250, 150,100,75,50,37,25,15,10 kDa) (Bio-Rad, CA) was added to each SDS-PAGE gel run.

2.3.7. Western-Blot analysis

Samples for Western blot analysis were first subjected to a 12.5% SDS-PAGE, followed by a transfer onto a polyvinylidene fluoride (PVDF) membrane using a dry-blot method (Invitrogen, CA). The membrane was then blocked for 1 hour at room temperature with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TTBS). Primary antibodies were prepared by diluting the antibody with TTBS. The primary antibodies were mouse anti-MBP (1:10,000, New England Biolabs Inc., MA) and goat anti-FST (1:5,000, R&D Systems, MN). Membranes were incubated with the respective primary antibodies either at room temperature for 1 hour (anti-MBP) or
overnight at 4°C (anti-FST). The membrane was then washed three times (10 minutes for each wash) with TTBS. The membrane was then incubated with either 1:10,000 alkaline phosphatase conjugated anti-mouse IgG (anti-MBP samples) or 1:10,000 alkaline phosphatase-conjugated anti-goat IgG (anti-Fst samples) (Sigma, St. Louis, MO) in TTBS for one hour. After washing, the membrane was developed using BCIP/NBT substrate (nitrobluetetrazolium and bromo-cloro-indolyl phosphate from Pierce, Rockford, IL).

2.3.8. Amylose affinity purification of MBP-chFST315 and MBP-chFST315-Fc(3-4)

MBP-chFST315 and MBP-chFST315-Fc(3-4) recombinant proteins were purified using an amylose affinity resin (New England Biolabs Inc., MA). The crude supernatant cell extract was diluted with column buffer in a 1:5 ratio. The manufacturer recommends using 15 mL of amylose resin per 1 liter of E. coli cell culture. The amylose resin was first equilibrated with 100 mL of column buffer. The diluted crude extract was then applied to the column. The pass-through was collected at a rate of 1 mL/minute. The column was then washed with 100 mL of column buffer. Proteins bound to the column were then eluted with elution buffer (column buffer with 10 mM maltose).

2.3.9. Removal of maltose from affinity-elution fractions by hyroxyapatite chromatography

Hydroxyapatite resin (Bio-Rad, CA) was mixed (1:25 weight to volume ratio) with washing buffer (20 mM sodium phosphate, 200 mM NaCl, pH 7.2). After allowing the resin to settle, the washing buffer was removed. This was repeated additional two times. The resin was then placed into a 1.0 x 5.0 cm glass chromatography column.
Affinity purified protein samples were then loaded onto the hydroxyapatite column at a flow rate of 1 mL/minute. The resin was then washed with 200 mL of washing buffer. The MBP-chFST315 protein was then eluted with 20 mL of elution buffer (0.5 M sodium phosphate, pH 7.2).

Buffer exchange was performed using a 15 mL Amicon-ultra centrifugal protein concentrator (Millipore, MA). The eluted proteins were loaded onto the protein concentrator and centrifuged at 5,000g for 30 minutes to remove excess 0.5 M sodium phosphate. The final concentrated MBP-chFST315 protein was then diluted to 200 µg/mL with affinity column buffer.

2.3.10. Factor-Xa cleavage of MBP fusion protein from MBP-chFST315

Fifty µg of MBP-chFST315 (200 ng/mL) containing no maltose was mixed with 1 µg of factor-Xa (New England Biolabs Inc., MA). The digestion was then incubated overnight at room temperature. Cleavage was analyzed by SDS-PAGE gel electrophoresis. After confirmation of cleavage, recombinant proteins without MBP were purified by collecting the pass-through fractions in the amylose resin affinity column. Purifications of the chFST315 protein were analyzed by SDS-PAGE gel electrophoresis and western blot analysis.

2.3.11. Examination of MBP-chFST315 binding to myostatin and activin

Pull-down assay was performed to analyze the ability of MBP-chFST315 to bind to MSTN and activin (Fig. 6). One µg of myostatin (MSTN) or activin (R&D Systems, MN) was mixed with 3 µg of affinity purified MBP-chFST315 containing no maltose. The protein mixture was then mixed for 2 hours at room temperature with 50 µL of
amylose resin equilibrated with column buffer in a centrifugal spin column (Pierce, Rockford, IL). The resin was then centrifuged at 4,000 g for 1 minute. After removing the pass-through the resin was washed with 1 mL of column buffer. Centrifugation and washing was repeated additional two times. The resin was then mixed with 10 µL of 10% SDS and 5 µL of non-reducing SDS-PAGE loading buffer. After heating the sample at 100°C for 5 minutes, the loading buffer was carefully removed from the resin. The eluted protein was then analyzed by SDS-PAGE and western blot analysis.

2.3.12. Bioactivity test for the MBP-chFST315 protein MBP-chFST313 by pGL3-(CAGA)₁₂ Luc-luciferase reporter system

A204 rhabdomyosarcoma cells (ATCC, HTB-82) was seeded in a 96 well plated at 30,000 cell/well in DMEM media (Invitrogen, CA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin plus fungizone. Cells were maintained at 37°C and 5% CO₂. When the cells reached 50-70% confluence each well was transfected with 0.1 µg of pGL3-(CAGA)₁₂-luciferase (Luciferase) and 0.05 µg of pRL-TB (Renilla) plasmid (Promega, WI) using the FuGENE6 transfection reagent system (Roche, Mannheim, Germany). After 24 hours, the medium was replaced with serum-free DMEM and was incubated for 9 hours before protein treatment. Next, mouse MSTN (0.9 ng/well) (R&D Systems, Minneapolis, MN) and various concentrations of FST (R&D Systems, Minneapolis, MN), and MBP-chFST315 were added to the respective wells. Firefly and renilla activity was measured 9 hours later by adding cell luciferase/renilla substrate (Promega, Madison, WI) after cell lysis using a microplate-luminometer (Turner Biosystems Inc., CA).
Figure 5. DNA constructs for MBP (Blank pMAL-p5x/c5x plasmid), pMAL-p5x/c5x-chF315, and pMAL-p5x/c5x-chF315-Fc(3-4).
Figure 6. Diagram representing the steps performed in the MSTN/Activin pull down assay. (1) Amylose resin was placed onto a spin column and equilibrated with column buffer. (2) After incubating the resin with either MBP-chFST315 and ligand or ligand only for 2 hours at room temperature, (3) the column was washed with column buffer, and (4) eluted with 10% SDS.
2.4. RESULTS

2.4.1. Insertion of chFST315 and chFST315-Fc(3-4) fragments into pMAL expression plasmid

Chicken IgY constant domain 3-4 DNA fragment (Fc(3-4)) and chicken follistatin 315 DNA fragment (chFST315) were successfully constructed (Appendix 8 and 9 respectively). Fig. 7 shows the ligation result of chFST315 and chFST315-Fc(3-4) into pMAL-c5x/p5x expression plasmids. Ligation resulted in an increase in DNA size between each construct, suggesting a successful insertion of the DNA fragments. The digestion of chFST315-Fc(3-4)-pMAL-c5x plasmid with HindIII and SalI released a fragment with the size of Fc(3-4) (Fig. 8), supporting the successful insertion of the chFST315-Fc(3-4) DNA fragment. DNA sequencing of the chFST315-pMAL-c5x (Appendix 14) and chFST315-Fc(3-4)-pMAL-c5x plasmid (Appendix 15) further confirmed the correct insertion of the two fragments.

2.4.2. Periplasmic expression of MBP-chFST315, and MBP-chFST315-Fc(3-4) proteins

Expressions of MBP-chFST315 and MBP-chFST315-Fc(3-4) proteins were induced by the addition of IPTG for 4 hours in the NEB-express E. coli culture transformed with chFST315-pMAL-p5x and chFST315-Fc(3-4)-pMAL-p5x, respectively. The expressed MBP-chFST315 and MBP-chFST315-Fc(3-4) were expected to contain a N-terminal 42.5 kDa maltose binding protein (MBP) as a fusion partner from the cloning vector, thus the expected molecular size of MBP-chFST-315 and MBP-
chFST315-Fc(3-4) were 80 kDa, and 105 kDa, respectively. SDS-PAGE analysis of the induced *E. coli* cells showed the presence of expected protein bands (Fig. 9).

The solubility of the recombinant proteins was examined by SDS-PAGE analysis of soluble and insoluble fractions separated by centrifugation of cell lysates (Fig. 10). While MBP (42.5 kDa) was mostly expressed as a soluble form, both MBP-chFST315 (80kDa) and MBP-chFST315-Fc(3-4) (105kDa) were expressed in insoluble forms.

The pMAL-p5x expression system directs the expression of the recombinant protein to the periplasmic space of *E. coli*. Thus, it was questioned whether the periplasmic region might contain some soluble recombinant proteins. The periplasmic protein fraction was isolated by inducing osmotic shock. MBP expressed by the blank pMAL-p5x vector was observed in the periplasmic fraction (Fig. 11), confirming that the MBP protein was expressed in the periplasmic space. However, little MBP-chFST315 and MBP-chFST315-Fc(3-4) was observed in the periplasmic fraction.

2.4.3. Cytoplasmic expression of MBP-chFST315 and MBP-chFST315-Fc(3-4) in the *SHuffle*® *E.coli* system

Since the MBP-chFST315 and MBP-chFST315-Fc(3-4) were not expressed in soluble forms in the periplasmic expression system, we examined the expression of MBP-chFST315 and MBP-chFST315-Fc(3-4) proteins in the cytoplasm of *SHuffle*® *E. coli* strain, an engineered strain to help complex internal disulfide bond formation of proteins in the cytoplasm (Appendix 10). Fig. 12 shows the result of the SDS-PAGE analysis of soluble and insoluble expression of MBP-chFST315 in the *SHuffle*® strain. In *NEB-Express* strain, the MBP-chFST315 protein was expressed entirely in an insoluble
form, but in the *Shuffle*® strain, the MBP-chFST315 proteins was mostly in a soluble form. The MBP-chFST315 showed affinity to both the anti-FST and anti-MBP antibodies (Fig. 13), indicating a correct translation of the recombinant protein.

Fig. 14 shows the results of the SDS-PAGE analysis of soluble and insoluble expression of MBP-chFST315-Fc(3-4) in the *Shuffle*® strain. Like the expression of MBP-chFST315, the MBP-chFST315-Fc(3-4) protein was expressed entirely in an insoluble form in NEB-Express strain, but in the *Shuffle*® strain, the MBP-chFST315-Fc(3-4) protein was mostly in a soluble form.

The heavy chain Fc(3-4) domain of chicken IgY contains 5 cysteine residues (Appendix 7), and one of the cysteine participate in intermolecular disulfide bond formation. To examine the intermolecular disulfide bond formation of MBP-chFST315-Fc(3-4), the MBP-chFST315-Fc(3-4) protein was subjected to SDS-PAGE (8% SDS-PAGE gel) under reducing and non-reducing conditions (Fig. 15). Under reducing conditions, MBP-chFST315-Fc(3-4) migrated to the expect protein size (105 kDa). If the Fc(3-4) domain of the recombinant protein formed dimer under non-reducing conditions, a protein band around 210 kDa would have been observed. However, under non-reducing conditions, the MBP-chFST315-Fc(3-4) band was not observed, indicating that MBP-chFST315-Fc(3-4) did not form a dimer but rather a multimeric protein complex consisting of multiple MBP-chFST315-Fc(3-4) proteins.

**2.4.4. Amylose resin affinity purification of MBP-chFST315 and MBP-chFST315-Fc(3-4)**
The pMAL-c5x system fuses a MBP to the N-terminal of target proteins. The MBP fusion protein can be used to immobilize fusion proteins to an amylose matrix. After removing the impurities, the fusion protein can then be eluted by the addition of maltose. Fig. 16 shows the result of SDS-PAGE analysis of the affinity purified MBP-chFST315 protein under reduced conditions. Compared to the crude extract (lane 2), the protein bands from the eluted fractions are predominantly that of MBP-chFST315 (lane 4). About 14 mg of affinity-purified MBP-chFST315 were harvested per 1 liter of *E. coli* culture (Table 2).

Fig. 17 shows the result of SDS-PAGE analysis of the affinity purified MBP-chFST315-Fc(3-4) under reduced conditions. Multiple protein bands ranging from 37-105 kDa was observed in the eluted fraction. Western blot analysis was performed to identify the multiple protein bands. The MBP-chFST315-Fc(3-4) protein contains a heavy chain constant domain which can be recognized by anti-chicken antibody, thus we performed a western blot using only anti-chicken-IgY-alkaline conjugated antibody as the primary antibody. Fig. 18 shows the Western blot result using either anti-chicken-IgY antibody (B), or anti-Fst antibody (C). A single protein band at the expected size of MBP-chFST315-Fc(3-4) was observed in anti-IgY blot. However, anti-FST antibody blot showed multiple protein bands corresponding to the unidentified protein bands observed in the SDS-PAGE gel. The multiple bands observed in the SDS-PAGE gel may have been degradation products of the recombinant protein.

2.4.5. Factor-Xa cleavage of MBP-chFST315 and purification of chFST315
To facilitate the removal of MBP fusion partner from target proteins, factor-Xa cleavage site (Ile-Glu-Gly-Arg) was inserted between the MBP and chFST315 of MBP-chFST315 protein. To obtain chFST315 without MBP fusion partner, affinity-purified MBP-chFST315 was incubated overnight with factor-Xa. SDS-PAGE analysis under non-reducing condition showed that the MBP-chFST315 protein band (80kDa) completely disappeared after overnight incubation along with the appearance of MBP (42.5 kDa) band, and factor-Xa (50 kDa), but the chFST315 band (36 kDa) was not clearly visible in the Coomassie-stained gel (Fig. 19). Western blot analysis showed that the 42.5 kDa protein had affinity only to anti-MBP antibody (Fig. 16B), supporting the separation of the MBP from MBP-chFST315-Fc(3-4). Western blot analysis with anti-FST antibody showed that after digestion, a protein band of around 37 kDa had affinity to anti-FST antibody, indicating that chFST315 was released from the MBP-chFST315 by factor Xa digestion (Fig. 19C). Undigested MBP-chFST315 and MBP can be removed from the sample by an additional round of amylose affinity chromatography. However, maltose needs to be removed from the buffer before affinity chromatography. After removing maltose by hydroxyapatite chromatography, the factor Xa-cleaved sample was reapplied to the amylose affinity matrix, and pass-through fraction was collected to obtain chFST135. SDS-PAGE and Western blot analysis showed that majority of the MBP and MBP-chFST315 protein was removed by the affinity chromatography (Fig. 19A).

2.4.6. Myostatin pull-down assay

To examine the binding of MBP-chFST315 to myostatin (MSTN), we performed a pull-down assay. Fig. 20 shows the results of the MSTN pull-down assay. MSTN by itself was incapable of binding to the amylose resin (data not shown). However, when
immobilized MBP-chFST315 was mixed with MSTN, the MSTN remained in the eluted fraction, indicating the binding of MSTN to MBP-chFST315.

MSTN and activin are both binding partners of FST, and it has been previously demonstrated that FST315 has a low binding affinity to activin in comparison to MSTN (Sugino et al., 1993; Cash et al., 2009). Therefore, we performed a pull down assay with MSTN and activin to determine qualitatively if MBP-chFST315 had lower binding affinity to activin. Activin (1 µg, Lane 1, 25 kDa) was mixed with MBP-chFST315 (3 µg), and in a separate tube, the same amount of MSTN (Lane 6, 25 kDa) was mixed with MBP-chFST315. Fig. 21 shows the result of SDS-PAGE analysis of the pull down assay with activin and MSTN. Activin by itself was not able to bind to the column (Lane 3). Similar to MSTN, when activin was mixed with immobilized MBP-chFST315, the activin molecule remained in the eluted fraction (Lane 4). However, despite the fact that equal amounts of each ligand was added during pull down assay, the amount of activin bound to MBP-chFST315 appeared to be lower than MSTN bound to MBP-chFST315 (Lane 7) based on visual evaluation of color intensity of the bands stained by Coomassie Blue. This suggests that MBP-chFST315 has a lower binding affinity to activin in comparison to MSTN.

2.4.7. Bioactivity test for the MBP-chFST315 protein using CAGA-Luciferase gene reporter assay

Next, we tested MSTN inhibitory capability of MBP-chFST315 utilizing a CAGA-Luciferase gene reporter assay. MSTN signals through a combination of type-II receptor (ActRIIB or ActRIIA) and type-I receptor (Alk4 or Alk5), which then activates a
SMAD signaling cascade (Rebbapragada et al. 2003). In the CAGA-Luciferase gene reporter assay, human A204 rhabdomyosarcoma cells are treated with MSTN. Activation of the SMAD signaling cascade induces the translation of a luminescent protein called luciferase. The level of MSTN signaling is represented by the luminescence given off as a result of substrate binding to the luciferase protein. Renilla was used as a transfection control. Complete inhibition of MSTN (9 ng/mL) activity was observed when treated with either FST315 expressed in eukaryotic system (R&D Systems, MN) or MBP-chFST315 at 500 ng/mL and 166 ng/mL, respectively (Fig 22, 23 respectively). Some inhibition (about 40%) of MSTN activity was observed at 55 ng/mL and 18.52 ng/mL in commercial FST315 expressed in a eukaryotic system. However, at similar levels, MBP-chFST315 was no longer able to inhibit MSTN, suggesting a potential difference in their affinity to MSTN between the MBP-chFST315 and FST315 expressed in a eukaryotic system.
2.5. Discussion

*Escherichia coli* remain a popular choice for recombinant protein production due to its relative low cost and high yields (Chou, 2007). Recombinant protein expression in *E. coli* is typically produced in the inner membranous cytosolic space. This creates a challenge to those who want to produce recombinant proteins with one or more disulfide bonds, as the reduced environment of the cytosol does not promote disulfide bond formation (Stewart et al., 1998). The periplasmic space of wild type *E. coli* is in oxidizing condition, thus structural disulfide bond formation is possible (Tan and Bardwell, 2004). In the periplasm, very strong oxidoreductase called DsbA facilitates the formation of disulfide bond by acting as an electron acceptor (Tan and Bardwell, 2004). Since Fst contains multiple number of intramolecular disulfide bonds, we attempted to produce the chFST315 and chFST315-Fc(3-4) in the periplasmic space using a vector designed to express recombinant proteins in the periplasmic space. Our current results demonstrate that the majority of our recombinant proteins (MBP-chFst315 and MBP-chFst315-Fc(3-4) formed insoluble inclusion bodies while MBP was able to be co-translated and translocated into the periplasmic space (Fig. 11). It has been shown that DsbA oxidizes consecutive cysteine residues as they are translocated into the periplasm, but proteins with a complex non-consecutive disulfide bonding patterns are incompatible for periplasmic translocation (Gentz et al., 1988; Tan and Bardwell, 2004; Ruiz et al., 2010). An estimated 14 and 16 nonconsecutive disulfide bonds is expected to form in chFST315 and chFST315-Fc(3-4), respectively (Fig.4). It is, thus, speculated that the presence of multiple nonconsecutive disulfide bonds in MBP-chFST315 and chFST315-Fc(3-4)
prevented the periplasmic expression of these proteins under the pMAL-p5x system in this study.

Recently, an *E. coli* strain has been genetically engineered to help facilitate the formation of disulfide bonds in the cytoplasm, and commercially named *SHuffle*® (Lobstein et al., 2012). This strain is unique in that it contains a nonfunctional thioredoxin-reductase-B (trxB), and is also genetically altered to overexpress DsbC, a disulfide-bond isomerase, in the cytoplasm (Lobstein et al., 2012). DsbC, a disulfide-bond isomerase protein, aids in proper folding of proteins with disulfide bonds in the periplasm (Tan and Bardwell, 2004). Thioredoxin super-family proteins are commonly found in the cytoplasm of plants, bacteria, and mammals (Collet and Messens, 2010). In the oxidized state, thioredoxin catalyzes the formation of disulfide bonds, but thioredoxin remains in the reduced state due to the presence of trxB in the cytoplasm of *E. coli* (Stewart et al., 1998; Lobstein et al., 2012). A recent study demonstrates a trxB loss of function (*FÅ113*) *E. coli* strain has the ability to form proteins with disulfide bonds in the cytoplasm of *E. coli* (Stewart et al., 1998; Ritz et al., 2001). Co-expression of DsbC in the cytoplasm of (*FÅ113*) *E. coli* (*SHuffle*®) further increased the yield of correctly folded disulfide bond-containing proteins in the cytoplasm (Lobstein et al., 2012). Consistent with the above result, MBP-chFST315 and MBP-chFST315-Fc(3-4) was produced in the soluble form in the *SHuffle*® *E. coli* strain in this study. This suggests that correct disulfide bond formation in Fst is integral in the proper folding of Fst. We further investigated if the solubility of MBP-chFST315 was due to the fusion of chFST315 to MBP a fusion partner commonly used to enhance the solubility of recombinant proteins. Removal of the MBP fusion protein by factor-Xa cleavage did not alter the solubility of
MBP-chFST315, suggesting that chFST315 was properly folded. One-step affinity purification of MBP-chFST315 gave nearly 14 mg of recombinant protein. However, substantial amount of MBP-chFST315 remained in the pass-through fraction during the first round affinity purification step. This might be due to the presence of a non-ionic detergent in the lysis buffer, which interfered with MBP binding to the affinity resin.

When the pass-through fraction was re-applied to the affinity column, additional 13 ± 1.37 mg per liter of culture of MBP-chFST315 could be obtained, resulting in the total yield of 27 ± 2.7 mg of recombinant MBP-chFST315 per liter of E. coli culture.

For the MBP-chFST315 to be a useful therapeutic agent, it should have biological activity. In a pull down assay, we observed the binding of MBP-chFST315 to MSTN as well as to activin, indicating a proper folding of the MBP-chFST315. Furthermore, concentration of 166 ng/mL of MBP-chFST315 protein was able to completely inhibit 9 ng/mL of MSTN signaling in an in vitro gene reporter assay, demonstrating its ability to suppress MSTN bioactivity. Fst315 has been previously demonstrated to have a greater binding affinity toward MSTN in comparison to activin (Nakatani et al., 2008b). The result of current pull down assay tend to support the above result because the amount of MSTN bound to MBP-chFST315 appeared to be greater than the amount of activin bound to the same unit of MBP-chFST315 when estimated by a visual evaluation of the band intensity stained by Coomassie blue.

Fc fusion proteins are proteins in which the Fc domain of a immunoglobulin is fused genetically with a protein of interest (Huang, 2009). The Fc domain of the fusion protein is thought to increase the serum half-life, thus increasing the therapeutic duration of the recombinant protein when administered (Huang, 2009; Strohl and Knight, 2009).
Therefore, we fused the Fc(3-4) portion of chicken IgY to the C-terminal of MBP-chFST315. The MBP-chFST315-Fc(3-4) protein band migrated to the expected protein size of 105 kDa under reduced condition (Fig. 15). Furthermore, the MBP-chFST315-Fc(3-4) showed to have affinity towards anti-chicken IgY antibody, suggesting that some anti-body like properties still remained (Fig. 18). The Fc(3-4) portion is expected to have 5 cysteine residues of which four cysteine participate in intramolecular disulfide bonds, and one cysteine participates in an intermolecular disulfide bond (Parvari et al., 1988). Therefore, the MBP-chFST315-Fc(3-4) monomer is expected to form a homo-dimer under unreduced condition. However, expression of MBP-chFST315-Fc(3-4) in ShufflE® E. coli resulted in a protein that is undetectable in an 8% SDS-PAGE gel under unreduced condition (Fig. 15). If MBP-chFST315-Fc(3-4) formed a dimer or a trimer the predicted protein sizes are 210 and 315 kDa respectively. However, the absence of the recombinant protein in a 8% SDS-PAGE gel (detection rage 20-350 kDa) suggest that the MBP-chFST315-Fc(3-4) formed a multimeric complex under unreduced condition.

Affinity purification and western blot analysis of MBP-chFST315-Fc(3-4) raised further questions on the proper folding of recombinant protein (Fig. 17). A single protein band was observed in the western blot analysis using anti-chicken-IgY as a primary antibody. However, anti-Fst western blot analysis resulted in multiple protein bands. This possibly suggest that MBP-chFST315-Fc(3-4) was degraded at the C-terminus, therefore the Fc(3-4) region would lose its affinity to the anti-chicken-IgY antibody.

In summary we have produced, for the first time, a recombinant, biologically active Fst315 (MBP-chFST315) molecule in E. coli. Moreover, we were able to purify mg levels of MBP-chFST315 from a 1 liter culture. We were also able to produce MBP-
chFST315-Fc(3-4) in the soluble form. Further investigations, however, need to be conducted to characterize the MBP-chFST315-Fc(3-4) protein. The ability to produce a large amount of Fst in a cost effective manner opens new avenues of research on investigating Fst potential to improve muscle growth.
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Table 2. Yield of MBP-chFST315 protein.

<table>
<thead>
<tr>
<th>Protein concentration estimated by modified Lowry protein assay</th>
<th>MBP-chFST315</th>
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<tr>
<td></td>
<td>Average yield per liter of culture (mg)</td>
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<tr>
<td>Total Protein</td>
<td>181.37 ± 7.81</td>
</tr>
<tr>
<td>Pellet Protein</td>
<td>8.54 ± 5.07</td>
</tr>
<tr>
<td>Soluble Protein</td>
<td>174.97 ± 22.1</td>
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<tr>
<td>Affinity Chromatography purified</td>
<td>14.04 ± 3.03</td>
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Percent yield of affinity purified MBP-chFST315 from 1 L *E.coli* culture was calculated as the mean ± SEM. The entire experiment was done in triplicates.
Figure 7. Insertion of chFST315 and chFST315-Fc(3-4) DNA fragments into either pMAL-p5x or pMAL-c5x vector.

chFST315-c5x/p5x plasmid and chFST315-Fc(3-4)-c5x/p5x plasmid was transformed into NEB-express E. coli. Plasmid DNA was extracted from E. coli culture using a single colony from each transformant. To compare the size of each construct, each plasmid was linearized with HindIII. Each sample were then electrophoresed and visualized under ultraviolet light. Kilo base pair (kbp), DNA ladder (L).
Figure 8. Agarose gel electrophoresis of chFST315-Fc(3-4)-c5x plasmid after restriction enzyme digestion. Fc(3-4) insert was released by digesting chFST315-Fc(3-4)-c5x with SalI and HindIII. Arrow indicates the Fc(3-4) DNA fragment. Kilo base pair (kbp), DNA standard ladder (L).
Figure 9. SDS-PAGE analysis of the expression of MBP (42.5 kDa), MBP-chFST315 (80 kDa), and MBP-chFST315-Fc(3-4) (105 kDa) in NEB-express E. coli transformed with the pMAL-p5x vector expression system. 100 µL equivalent of E. coli cell culture were subjected to SDS-PAGE gel electrophoresis. Arrows indicates the expected recombinant proteins. Protein expression before (U), and after (I) induction (0.4 mM IPTG at 30°C). Protein standard ladder (M).
Figure 10. SDS-PAGE examination of soluble and insoluble expression of MBP-chFST315 and MBP-chFST315-Fc(3-4) proteins. Expressions of MBP (empty vector), MBP-chFST315 and MBP-chFST315-Fc(3-4) were induced with the addition of IPTG (4h induction at 30°C with 0.4 mM IPTG). Cell lysis was carried out by adding lysozyme and DNase I to the E. coli cell pellet followed by pulse sonication. The total crude extract (T) was then centrifuged at 8,000 g for 30 minutes. The soluble supernatant fraction (S) was then separated from the insoluble fraction (P). Arrows indicates the expected recombinant proteins. 100 µL equivalent of E. coli cell culture were subjected to SDS-PAGE gel electrophoresis. Protein standard ladder (M).
Figure 11. SDS-PAGE examination of the periplasmic extraction of MBP (42.5 kDa), MBP-chFST315 (80 kDa), and chFST315-Fc(3-4) (105 kDa). Cells pellets were resuspended in 10 mL sucrose buffer (30 mM Tris, 20% sucrose,1 mM EDTA, pH 8.0). After pellting the cells once more the cell pellet was resuspended in 10 mL of 5 mM MgSO₄. The osmotic shock fluid which contains the periplasmic protein fraction was then obtaind by centrifugation at 8,000 g at 4°C for 30 minutes. Arrows indicate the expected recombinant proteins. 100 µL equivalent of *E. coli* cell culture were subjected to SDS-PAGE gel electrophoresis. Total crude extract (T), periplasmic protein (P), protein standard ladder (M).
Figure 12. SDS-PAGE examination of soluble and insoluble expression of MBP, and MBP-chFST315 protein utilizing the pMAL-c5x vector system. Expression of MBP, and MBP-chFST315 was induced with the addition of IPTG (4 hours induction at 30°C with 0.4 mM IPTG). Cell lysis was conducted by adding lysozyme and DNase I to the *E. coli* cell pellet followed by pulse sonication. The total crude extract (T) was then centrifuged at 8,000g for 30 minutes. The soluble supernatant fraction (S) was then separated from the insoluble fraction (P). 100 µL equivalent of *E. coli* cell culture were subjected to SDS-PAGE gel electrophoresis. Arrows indicate the expected MBP-chFST315 recombinant proteins. Protein standard ladder (M).
Figure 13. SDS-PAGE (A) and Western-Blot (B, C) analysis of MBP-chFST315 and commercially purchased FST (R&D Systems, MN). Affinity purified MBP-chFST315 (B, C), FST (B, C), and MBP (C) sample was transferred onto a PVDF membrane using a dry-transfer method (Invitrogen). The membrane was blocked with TTBS + 5% non-fat skim milk. The membrane was then incubated at room temperature for one hour with either (B) Goat affinity-purified polyclonal antibody against human follistatin (1:500) (R&D Systems, MN), or monoclonal anti-MBP (1:5,000) primary antibody. After washing, the membrane was incubated with either (B) anti-Goat alkaline-conjugated secondary antibody (1:10,000) or, (C) anti-mouse alkaline-conjugated secondary antibody (1:10,000).

Arrows indicate the expected recombinant proteins MBP-chFST315 (80 kDa), MBP (42.5 kDa). Protein standard ladder (M).
Figure 14. SDS-PAGE examination of soluble and insoluble expression of MBP-chFST315-Fc(3-4) protein utilizing the pMAL-c5x vector system. Expression of MBP-chFST315-Fc(3-4) was induced with the addition of IPTG (4h induction at 30°C with 0.4mM IPTG). Cell lysis was conducted by adding lysozyme and DNase I to the E. coli cell pellet, followed by pulse sonication. The total crude extract (T) was then centrifuged at 8,000 g for 30 minutes. The soluble supernatant fraction (S) was then separated from the insoluble fraction (P). 100 µL equivalent of E. coli cell culture were subjected to SDS-PAGE gel electrophoresis. Arrows indicate the expected MBP-chFST315-Fc(3-4) recombinant protein. Un-induced protein fraction (U), protein standard ladder (M).
Figure 15. SDS-PAGE examination of the MBP-chFST315-Fc(3-4) protein under reduced and non-reducing conditions. The total crude extract (T) was then centrifuged at 8,000 g for 30 minutes. The soluble supernatant fraction (S) was then separated from the insoluble fraction (P). An 8% SDS-PAGE under reduced and non-reducing condition was used to access the formation of MBP-chFST315-Fc(3-4) dimer. Arrows indicates the expected MBP-chFST315-Fc(3-4) recombinant protein under reduced conditions. Protein standard ladder (M).
Figure 16. SDS-PAGE analysis of affinity purification of MBP-chFST315. The soluble crude extract obtained from a 1 liter culture of *Shuffle*-MBP-chFST315 (S) was applied to 15 mL of amylose affinity matrix. The MBP-chFST315 binding affinity to the amylose resin was also assessed by analyzing the pass-through (P). Proteins immobilized to the amylose column were then eluted with an elution buffer containing maltose (E). Arrows indicate the expected affinity purified MBP-chFST315 protein. Protein standard ladder (M).
Figure 17. SDS-PAGE analysis of affinity purification of MBP-chFST315-Fc(3-4). The soluble crude extract obtained from a 500 mL culture of Shuffle®-MBPchFST315-Fc(3-4) (S) was applied to 15 ml of amylose affinity matrix. The MBP-chFST315-Fc(3-4) binding affinity to the amylose resin was also assessed by analyzing the pass through (Pass). Proteins immobilized to the amylose column were then eluted with an elution buffer containing maltose (E1-E3). Arrow indicates the expected affinity purified MBP-chFST315-Fc(3-4) protein. Protein standard ladder (M); Insoluble fraction (P).
Figure 18. SDS-PAGE (A) and Western-Blot (B, C) analysis of MBP-chFST315-Fc(3-4).

Affinity purified MBP-chFST315-Fc(3-4) (105 kDa, (E)) and total soluble extract (S) was transferred onto a PVDF membrane using a dry-transfer method (Invitrogen). The membrane was blocked with TTBS + 5% non-fat skim milk. The membrane was then incubated overnight at 4°C with either (B) Anti-chicken-IgY alkaline phosphatase conjugated antibody (1:5,000) (Sigma-Aldrich, St. Louis, MO), or anti-FST (1:500) primary antibody (R&D Systems, MN). After washing anti-FST Western-Blot samples were incubated with anti-goat alkaline-conjugated secondary antibody (C) (1:10,000). Since the Fc-(3-4) was used as a primary antibody, anti-chicken-IgY was developed without the addition of a secondary antibody. Protein standard ladder (M).
Figure 19. SDS-PAGE (A) and Western-Blot (B, C) analysis of chFST315 purification after factor-Xa cleavage of MBP-chFST315.

Affinity purified MBP-chFST315 (black arrow, 80 kDa, (Uₖ)) was digested overnight (Cut (C)) with factor-Xa (red arrow, 50 kDa, (Xa)) in order to separate MBP (yellow arrow, 42.5 kDa, (MBP)) and chFST315 (blue arrow, 35.7 kDa, (E)). The digested mixture was then loaded onto amylose resin. Protein which was not bound to the column was collected and analyzed by SDS-PAGE gel electrophoresis (E). Western-blot analysis with either anti-MBP (B) or anti-FST (C) was used to analyze the digestion. (FST) commercial FST (R&D Systems, MN).
Figure 20. SDS-PAGE analysis of MBP-chFST315 binding to MSTN in a pull-down assay. 50 µl of amylose resin was mixed with either 3 µg of MBP-chFST315 (Lane 3) or 1 µg of MSTN plus 3 µg of MBP-chFST315 (Lane 4). The column was then washed with column buffer multiple times. Proteins bound to the amylose resin was then eluted with non-reducing SDS-PAGE loading buffer and were subjected to SDS-PAGE analysis.

Lane 1, MBP-chFST315 (80 kDa); Lane 2, MSTN (25 kDa at non-reduced condition); Lane 3, MBP-chFST315 elution; Lane 4: MBP-chFST315 incubated with MSTN elution. Arrow indicates the MSTN protein band.
Figure 21. SDS-PAGE analysis of MBP-chFST315 binding to MSTN and activin in a pull-down assay. 50 µl of amylose resin was mixed with either 3 µg of MBP-chFST315 (F), 3 µg of MBP-chFST315 and 1 µg of activin (A+F), 3 µg of MBP-chFST315 and 1 µg of MSTN (M+F), or 1µg activin only (E). The column was then washed with column buffer multiple times. Proteins bound to the amylose resin was then eluted with non-reducing SDS-PAGE loading buffer and were subjected to SDS-PAGE analysis. Lane 1, Protein ladder; lane 2, activin (25 kDa under non-reduced condition); lane 3, activin elution; lane 4, MBP-chFST315 incubated with activin elution; lane 5, MBP-chFST315 elution (80 kDa); Lane 6 MSTN (25 kDa at non-reduced condition); Lane 7, MBP-chFST315 incubated with MSTN elution. Red arrow indicates the MSTN protein band; yellow arrow indicates the activin protein.
Figure 22. Effect of commercial Fst on MSTN signaling. A204 rhabdomyosarcoma cells (ATCC, HTB-82) was seeded in a 96 well plated at 30,000 cell/well in DMEM media (Invitrogen, CA). When the cells reached 50-70% confluence each well was transfected with 0.1 µg of pGL3-(CAGA)$_{12}$-luciferase (Luciferase) and 0.05 µg of pRL-TB (Renilla) plasmid (Promega, WI) using the FuGENE6 transfection reagent system (Roche, Mannheim, Germany). After 24 hours, the medium was replaced with serum-free DMEM and was incubated for 9 hours before protein treatment. Next, mouse MSTN (0.9 ng/well) (R&D Systems, Minneapolis, MN) and various concentrations of FST (R&D Systems, Minneapolis, MN), were added to the respective wells. Firefly and renilla activity was measured 9 hours later by adding cell luciferase/renilla substrate (Promega, Madison, WI) after cell lysis using a microplate-luminometer (Turner Biosystems Inc., CA). Renilla was used as a transfection efficiency control.
Figure 23. Effect of MBP-chFST315 on MSTN signaling. A204 cells were treated with MSTN (0.9 ng/well) (R&D Systems, Minneapolis, MN) and various concentrations of MBP-chFST315. Firefly and renilla activity was measured 9 hours later by adding cell luciferase/renilla substrate (Promega, Madison, WI) after cell lysis using a microplate-luminometer (Turner Biosystems Inc., CA). Renilla was used as a transfection efficiency control.
Appendix 1

Original chFST315 Sequence. The sequence included in the FST 315 insert is underlined.

ATGTTAAATCAGAGGATCCACCCGGGCATGCTCGTACTCCTGATGTTTCTCTA
CCACTTCATGGAAGATACACACAGCGCAGGCTGGGAATTGTTGGCTCCGGCCAG
GCGCGGAACGGCCGCTGCCAGGTCCCTCTCTACAAGACCAGACCTCAGGAAAGAG
GAGTGCTGCAAGAGCGCCTGACGCTCGTGGACGGAGGAGGACGGACGTC
AACGACAACACGCTCTTTAAGTGGATGATTTTTAATGGGGGAGGCCCCTAACT
GCATCCCCGTGCAAGAAACATGTGAAGATGTGGACTGTGGACCTGGGACTTG
CTCTAATATCACCTGGAAGGGCCCCCGTGTTGCTGGATGCTGATGGGAAAACCTAC
AGGAACGAGGTGTTGCCCCCTCTCAAAGCCAGATGAAGAACAGCAGCCCGAACTTG
AAGTCAAATATCCAGGGCAAGTGCAAAAAACCTGTAGGGATGTTTTATGGCC
AGGCAGCTCCACGTTGTTGATCAAACCTACGCTACTGTTGAGACA
TGTAATCGAATTGCCCCGCTGAGCCTACCCCTCCCTGAGCGATATCTTCTGAGA
TGATGGGCATAAACTTGCCAGCGCTGCCCTGAGCAGAAAAAGCGACCTGCTCTG
CTGGGCAGATCCATTGGATTAGCTACGGAAAAATGCACTCAAAGCGAAGTG
CCTGTGAAAGATAATTAGTCAGTGCAGTGGGATTGAAATGCTTGGGATTTTAA
GGTGGGGAAGGCCGATGCCTCTGTGACGCTCGCTGCGGCTGAAAGCAAG
TCAGATGAGGCAGCTCTGTGCCAGTGATAACACAACTTATCCGAGCGAGTG
CCATGAAAGGAGCGAGGCTGTTCCATGGGCCCGTGCTTCTAGAGTAAGTAAACGACTC
TGGATCTGGCAACTTACGCGAAGACCGAGGGAAGAAGGAGATGAGACCAGGACTACAGCTTCTCTATCCTATTAGAGTGGTA
Appendix 2

Optimized FST315 DNA sequence.

5’Smal site - CCCGGG -

GGTAACTGCTGGCTGCAGGCTCGTAATGGCCGTTGTCAAGTGCTGTATAA
AACCGATCTGTCTCCAAAGAAGATGCTGTAATCCCGGCCGTCTGACCACGTCA
TGGACCGAAGAAAGATGTAAACGACAATACGCTGTITTTAATGGATGATTTTCA
ACGCGCGGTGCAGCCGAATTGCATCCCGGTGAAAGGAAGACCTGCGAAAAACGTCGA
TTGTTGGTCCCGGGCAAAAAATGCAAATAATGAACAAGAAAAACAAACCGCGTTG
CGTGTGTCACCCGGACTGCTCACAACATTACCTGAAAAGGTCCCGGTITTTGTCGCC
TGGATGGTTAAAACGTATCGTAATGAATGCGCACTGCTGGAAGCTCGCTGTAAA
AGAACAGCCGGAACGTGAAGTTTCAGTACCAAGGAAATGCAAGAAAAACCTG
TCGTTGATGTCTCTGTGTCGGCGGCAGCTCTCACTGTGTTGTTGACCAGACGAACA
ATGCATATTGCGTGACCTGTAACCGCATTGCCCAGAACCAGCGTGCTGGCAGCTCA
ACAATATCTGTGTTGCCAATGTGGTATCACCAGCACAGCGCCATCTGC
GTAAAGCGACGTGTCGTTGGGCGCTCGATTGTTGCTGGCTACAGGAAGGCAA
ATGCATTAAAAAGCCAAAAAGCTGCGAAGATTATCCAAATTTCTGCAGGTAACAA
TTGCTGTGGGACTTTAAAAATCGGCCCGTGTCGTACAGCGGCCGTGTGATGAACT
GTGCCCGAAGGTAATCCCGATGAAACGGGTGCTGGCCAGTAGTGAATACGCC
TATCCGTCCGAAATGCGCCATTGAAGAAAGCGCCCTGTCGACTCGATGGGCCTCTGC
TGGAAGTGAAACACTCAGGTTGCTGCAACTCTATCATGAAAGATCAGGAAAG
AGAAGAAGAGATGAGGAAGCAAGACTACTCCTTTCGGATTTCAGCATCCTG
GAATGGCCG-GGT-GCC-GCC-CAC-TAT-

GTGCAC -3’ SalI site
Appendix 3

Modified nucleotides in the chFST315 mRNA sequence starting from the 5’ end of the N-terminal domain and 3’ end of the C-terminal domain

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Appendix 4

Chicken IgY Fc3-4 DNA sequence. Features of the Fc3-4 insert include a 5’SalI site and a 3’ HindIII site.

5’ SalI site-GTCGAC-

GACGGCGCTCAGAGCTGCAGCCCCATCCAGCTGTACGCGCATCCCACCAGCC
CGGGCGAGCTGTACATCAGCTTAGACGCCAACACTGAGGTGCCTGGTGGTCAA
CCTGCCCCAGCGATTTCCAGCTAGCGTACCTGGACCAGGGAGAAGAGTGGG
AACCTCCGGCCCGACCCGATGGGGGTCTCCAAGAACACTTCAACGGCACCTACA
GCGCCACAGCAGCGCCGTCCCCGTCAGCACCAGATTGTTATCCGGGGAGAG
GTTCACCTGCACCGTGACAGCAGGAGGCTGCCCTGCGCTGCTGCAAGAGC
GTCTACAGGAACACGGGACCCACCACCCACCTCTGATCTACCTCCCCTCGCCC
CCACCCGGAAGAGCTGTCCCTCTCCCGCGTCACCCTGAGCTGCCCTGCTGGTCCG
GCTTCCGCCACGCTGACATCGAGATCCCGCTGGGCTCCGGACCCACCAGCGCCGT
TCCCCGCCACCGAATTCTGCAACCACCGCCGTCTCCCGGAAGAGAGACCGCA
AACGGCGCCCGGGGCGTGACGCGACACTTCTTCGCTGTAACAGTAGATGACG
TGAGAACCAGCAAGTGGAACCGCGGAGCTGCTGCTGCATGGCGGTGCA
CGAGGCGGTGCCCATGCGCTTCAGCGCCAGCGACGCTGCGAGAAGAGGTGGT
AAA-

AAGCTT-3’ HindIII site
Appendix 5

chFST315-pMAL-p5x/c5x and chFST315-pMAL-p5x/c5x DNA constructs

(A) malE…

GGG
GGTAACTGCTGGCTGCGTCAGGCTCGTAATGGCCGTTGTCAAGTGCTGTATAAAACC 60
GATCTGTCCAAAAGAAGAATGCTGTAAATCCGGCCGTCTGACCACGTCATGGACCGAAGAA 120
GATGTTAACGACAATACGCTGTTTAAATGGATGATTTTCAACGGCGGTGCGCCGAATTGC 180
ATCCCGTGTAAAGAAACCTGCGAAAACGTCGATTGTGGTCCGGGCAAAAAATGCAAAATG 240
AACAAGAAAAACAAACCGCGTTGCGTGTGTGCACCGGACTGCTCCAACATTACCTGGAAA 300
GGTCCGGTTTGTGGCCTGGATGGTAAAACGTATCGTAATGAATGCGCACTGCTGAAAGCT 360
CGCTGTAAAGAACAGCCGGAACTGGAAGTTCAGTACCAAGGCAAATGCAAGAAAACCTGT 420
CGTGATGTCCTGTGTCCGGGCAGCTCTACCTGTGTGGTTGACCAGACGAACAATGCATAT 480
TGCGTGACCTGTAACCGCATTTGCCCGGAACCGACGAGCCCGGAACAATATCTGTGTGGC 540
AATGATGGTATCACCTACGCATCAGCTTGCCATCTGCGTAAAGCGACGTGTCTGCTGGGC 600
CGCTCGATTGGTCTGGCGTACGAAGGCAAATGCATTAAAGCCAAAAGCTGCGAAGATATC 660
CAATGTTCTCGAGTAAAAATCTCTGTGGGACTTTAAAGTCGGCCGTGGTCGCTGCGCT 720
CTGTGTGATGAACTGTGCCCGGAAAGTAAATCCGATGAAGCGGTGTGTGCCAGTGACAAT 780
ACCAGTATCCGTCCGGCGACGCTTACACTCTTTCCGATTTCAACGGCACCTACAGCGCCAGC 840
CACATGAAACACTCAGGTTCGTGCAACTCTATCAATGAAGATCCGGAAGAAGAAGAAGAA 900
GATGAAGACCAAGACTACTCCTTTCCGATTTCAAGCATCCTGGAATGGCCGGGTGCCGCC 960
GTCGAC 972

(B) malE…

GGG
GGTAACTGCTGGCTGCGTCAGGCTCGTAATGGCCGTTGTCAAGTGCTGTATAAAACC 60
GATCTGTCCAAAAGAAGAATGCTGTAAATCCGGCCGTCTGACCACGTCATGGACCGAAGAA 120
GATGTTAACGACAATACGCTGTTTAAATGGATGATTTTCAACGGCGGTGCGCCGAATTGC 180
ATCCCGTGTAAAGAAACCTGCGAAAACGTCGATTGTGGTCCGGGCAAAAAATGCAAAATG 240
AACAAGAAAAACAAACCGCGTTGCGTGTGTGCACCGGACTGCTCCAACATTACCTGGAAA 300
GGTCCGGTTTGTGGCCTGGATGGTAAAACGTATCGTAATGAATGCGCACTGCTGAAAGCT 360
CGCTGTAAAGAACAGCCGGAACTGGAAGTTCAGTACCAAGGCAAATGCAAGAAAACCTGT 420
CGTGATGTCCTGTGTCCGGGCAGCTCTACCTGTGTGGTTGACCAGACGAACAATGCATAT 480
TGCGTGACCTGTAACCGCATTTGCCCGGAACCGACGAGCCCGGAACAATATCTGTGTGGC 540
AATGATGGTATCACCTACGCATCAGCTTGCCATCTGCGTAAAGCGACGTGTCTGCTGGGC 600
CGCTCGATTGGTCTGGCGTACGAAGGCAAATGCATTAAAGCCAAAAGCTGCGAAGATATC 660
CAATGTTCTCGAGTAAAAATCTCTGTGGGACTTTAAAGTCGGCCGTGGTCGCTGCGCT 720
CTGTGTGATGAACTGTGCCCGGAAAGTAAATCCGATGAAGCGGTGTGTGCCAGTGACAAT 780
ACCAGTATCCGTCCGGCGACGCTTACACTCTTTCCGATTTCAACGGCACCTACAGCGCCAGC 840
CACATGAAACACTCAGGTTCGTGCAACTCTATCAATGAAGATCCGGAAGAAGAAGAAGAA 900
GATGAAGACCAAGACTACTCCTTTCCGATTTCAAGCATCCTGGAATGGCCGGGTGCCGCC 960
GTCGAC 972

DNA sequences of chFST315 (A) and chFST315-Fc(3-4) (B). Both DNA fragments were subcloned into the 5’ XmnI and either 3’ SalI (chFST315 fused with Fc(3-4)) or 3’ HindIII (chFST315 only) restriction enzyme sites (Restriction enzyme sites are highlighted). Underlined sequences correspond to Genenase I proteolytic site. The correct construct was confirmed by DNA sequencing analysis (EWG Operon, CA)
Appendix 6

Amino acid sequence of MBP-chFST315 protein. The MBP amino acid sequence is underlined.

MKIEEGKLVWNGDKGYNGLAEVGKKEKDTGKVTVEHPDKLEEKFQPQVAAT
GDGDIIIFWADDRFAGYQAQGLLAEITPDKAFQDKLYPFTWDARVYNKLIAYPI
AVEALSIYKDLPLNPPTWEEIPALDKELKAKGKSMFNLQEPYFTWPLIAA
DGGYAFKYENGKYDIDGVGDNAGAKAGLTFLVDLILKNKHMADTDYAESIAAA
FNKGETAMTINGPWAWSNIDTSKVNMYGTVALPTFKGQPSPFVGVLGAINAAS
PNKELAKEFLENYLTLDEGLEAVNKDKPLGALAVKSYEELVTKDRIAAATMENA
QKGEIMPNIQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSSSNNNNNN
NNNLGIEGRISHMSMGRDIVGSEFPAGNGNCWLRQARNGRCQVLYKTDLS
KEECKSGRLTTSWTEEDVNDNTLFKWMIFNNGAPNCIPCKETCENVDGPGKK
CKMNKK_KPVCAPDCSNITWKGPGCLGKTYRNECALLKKRRCEQFPELEV
QYQGKCKKTKCDVLCPGGSTCVVDQTNAYCVTNICPEPTSPEQYLCNMDGI
TYASACHLRKATCLLGRSGLAYEGKCIKAKSCEDIQCSAGKCKLWDFKVGRGR
CALCDELCPESKSDEAVCASDNTTYPSECAMKEAACSMSGYLVVEKHSKSCNSIN
EDPEEEEDEDQDYSFPISSILEWPGAAHYVD
Appendix 7

Amino acid sequence of MBP-chFST315-Fc(3-4) protein. The MBP amino acid sequence is underlined. Fc(3-4) amino acid sequence is highlighted.

MKIEEGKLVIWINGDGKYNGGLAEVGKKFEKDTGIKVTEHPDKLEEKFQPVAAT
GDGPDIIFWAHDFGGYAQSGLLEAITEPDKAFOQDKLYPEFTWAVRYNGKLIAYPI
AVEALSLIYNDLLPNNPPTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAA
DGGYAFKYENGKDYIKDVGVDNAGAKAGLTLVLKIKNKHMNADTDYSIAEEA
FDNGKGETAMTINGPWAWNIDTSKVNYGVTVPFTFGQPSKPFGVLSAGINAAS
PNKELAKEFLENYLLTDLEGLEAVNKDKPLGAVALKSYEELVKEPRIATMENA
QKGEIMPNIPOMSAFWYAVRTAVINAAASGRQTVDEALKDAQTNSSSNNNNNN
NNNLGIEGRISHMSMGGRDIVDGSEFPAGNGNCWLQRARNGRCQVLYKTDLS
KEECCKSGRTTEWSWEEDVNDNTLFKMIFNGGAPNCPCETCENVDCGPGKK
CKMNKKNPRLCVCAPDCSNITWKGPVCGLDGKTYRNECALLKARCEQEPELEV
QYQGKCKKTCDVLCPSSTCVDQTNNAYCVTCNRCPEPTSPEQLCGNDGI
TYASACHLRKATCLLGRSISLAYEGKCIKAKSCEDIQCSAGKCLWLDFKVGRGR
CALCDECPESKSDEAVCASDNTTYPSECAMKEAACSMDGFLLEVKHSGSCNSIN
EDPEEEEEDEQDSPISSILEWPGAAHYVDDGAQSCSPIQLYAIPPSPEGLYISL
DAKLRCVLVNLPSDSLSTVTREKSGNLRPDPMVLOEHFNGTYSASSAVPST
QDWSGERFTCTVQHEEPLSLKSVYRTGTTPPLIYPFAPHPEELSRLVRVTLSC
LVRGFRPRDIEIRWLRDHRAPATEFVTTAVLPEERTANGAGGDGDFTVYSKM
SVETAKWNGGTGFACMAVHEALPMRFSQRTLQKQAGKKL
Appendix 8

A)

B)

Agarose gel electrophoresis of DNA fragments obtained from polymerase chain reaction of (A) chicken IgY FC domain 3-4 (681 bp) amplified from chicken spleen cDNA library and (B) chicken IgY FC domain 3-4 containing 5’Sall and 3’HindIII (693 bp) amplified from chicken IgY FC domain 3-4 amplicon. A standard PCR thermo-cycling program with annealing temperatures of 55.6 °C and 56.6°C was used to amplify the desired DNA fragment. DNA standard ladder (L).
Appendix 9

Agarose gel electrophoresis of chFST-315-pUC-57 after restriction enzyme digestion of chFST-315-pUC 57 plasmid. The codon-optimized chFST315 DNA sequence (975 bp) containing a 5’ SmaI and 3’ SalI restriction site was commercially synthesized and cloned into a pUC-57 plasmid. The chFST-315-pUC-57 plasmid (2.8 Kbp) was digested with SmaI and SalI. The digested and undigested samples were electrophoresed and visualized under ultraviolet light. Arrows indicates the chFST315 insert. DNA standard ladder (L).
Appendix 10

SDS-PAGE examination of MBP, and MBP-chFST315 expressed in either *Shuffle*® or *NEB-express* *E.coli* using the pMAL-c5x vector system. *Shuffle*® was transformed with either blank pMAL-c5x plasmid or chFST315-c5x plasmid. *NEB-express* was only transformed with chFST315-c5x plasmid. Cell pellets collected from 100 ml amount of *E. coli* cell culture were subjected to SDS-PAGE gel electrophoresis. Before induction (U), after induction (0.4 mM IPTG at 30°C) (I). Arrows indicate the expected MBP-chFST315 recombinant proteins. Protein standard ladder (M).
Appendix 11

SDS-PAGE analysis of affinity purified MBP-chFST315. The soluble crude extract (S) was applied to an affinity matrix of amylose. The column was then washed with column buffer to remove unbound proteins (P1). A protein immobilized to the column was eluted with an elution buffer containing maltose (E1). After regeneration of the amylose column, (P1) was reapplied to column. The column was then washed (P2), and eluted (E2) as previously described. The entire process was repeated two additional times (P3-4; E3-4).

MBP-chFST315 was also observed in the pass through fraction. This may be caused by multiple factors. Overloading of the amylose column beyond the recommended capacity is one example. However, the 17 mg of recombinant protein purified (Table 2) is
below the binding capacity expected per 15 ml of amylose resin. According to the manufacture, 15 ml of amylose resin is capable of binding to 45 mg of recombinant MBP. A second consideration is that the buffer used to lyse the cells contained ionizing detergents. According to the manufacture, the presence of ionizing detergents may interfere with MBP binding to the amylose matrix. Damage of the MBP during the expression and folding process may also diminish binding capabilities.

Since some MBP-chFST315 protein remained in the pass-through, it was questioned whether the MBP-chFST135 was capable of binding to the amylose column. When reapplying the pass through to the newly regenerated amylose matrix, MBP-chFST315 was able to bind to the amylose resin. After repeated rounds of affinity purification, we were able to extract an additional 13 mg of MBP-chFST315.
Appendix 12

**BSA STANDARD CURVE**

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* Goodness of fit value $R^2=0.9964$
Appendix 13

Total yield of MBP-chFST315 were estimated based on BSA standard curve

(Appendix 12)

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Appendix 14

DNA sequencing of pMAL-c5x-chFST315 plasmid using the malE sequencing primer set (New England Biolabs Inc, MA)

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# Appendix 15

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</tr>
<tr>
<td>951-1000</td>
<td>GATGAAGACC AAGACTACTC CTTTCCGATT TCAAGCATCC TGGAATGGCC</td>
<td>GATGAAGACC AAGACTACTC CTTTCCGATT TCAAGCATCC TGGAATGGCC</td>
<td>GATGAAGACC AAGACTACTC CTTTCCGATT TCAAGCATCC TGGAATGGCC</td>
</tr>
<tr>
<td>1001-1050</td>
<td>AGCTGTACGC CATCCC ACCTG GACGGCCAG AGCTGTACAT CAGCTTAGAC</td>
<td>AGCTGTACGC CATCCC ACCTG GACGGCCAG AGCTGTACAT CAGCTTAGAC</td>
<td>AGCTGTACGC CATCCC ACCTG GACGGCCAG AGCTGTACAT CAGCTTAGAC</td>
</tr>
</tbody>
</table>
chFST315Fc(3-4) SEQ.
AGCTGTACGC CATCCCCACC AGCCCCGGCG AGCTGTACAT CAGCTTAGAC
Chromatid

chFST315Fc(3-4) OR.
GGCCAAATCGA GTGTCGCTGTT GTGCAACCTG CCGACGGTAT CATGCTCTCAG
Chromatid

chFST315Fc(3-4) SEQ.
GCCAAATCGA GTGTCGCTGTT GTGCAACCTG CCGACGGTAT CATGCTCTCAG
Consensus

Chromatid

chFST315Fc(3-4) OR.
CGACGGAGA GTGCCCGCTGC CGCTCAGCAA GAGCGTCTAC AGGAACACGG
Chromatid

chFST315Fc(3-4) SEQ.
CGACGGAGA GTGCCCGCTGC CGCTCAGCAA GAGCGTCTAC AGGAACACGG
Consensus

Chromatid

chFST315Fc(3-4) OR.
CGACGGAGA GTGCCCGCTGC CGCTCAGCAA GAGCGTCTAC AGGAACACGG
Chromatid

chFST315Fc(3-4) SEQ.
CGACGGAGA GTGCCCGCTGC CGCTCAGCAA GAGCGTCTAC AGGAACACGG
Consensus

Chromatid

chFST315Fc(3-4) OR.
CGACGGAGA GTGCCCGCTGC CGCTCAGCAA GAGCGTCTAC AGGAACACGG
Chromatid

chFST315Fc(3-4) SEQ.
CGACGGAGA GTGCCCGCTGC CGCTCAGCAA GAGCGTCTAC AGGAACACGG
Consensus

Chromatid

chFST315Fc(3-4) OR.
CGACGGAGA GTGCCCGCTGC CGCTCAGCAA GAGCGTCTAC AGGAACACGG
Chromatid

chFST315Fc(3-4) SEQ.
CGACGGAGA GTGCCCGCTGC CGCTCAGCAA GAGCGTCTAC AGGAACACGG
Consensus

Chromatid

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CGACGGAGA GTGCCCGCTGC CGCTCAGCAA GAGCGTCTAC AGGAACACGG
Chromatid

chFST315Fc(3-4) SEQ.
CGACGGAGA GTGCCCGCTGC CGCTCAGCAA GAGCGTCTAC AGGAACACGG
Consensus

Chromatid

chFST315Fc(3-4) OR.
CGACGGAGA GTGCCCGCTGC CGCTCAGCAA GAGCGTCTAC AGGAACACGG
Chromatid

chFST315Fc(3-4) SEQ.
CGACGGAGA GTGCCCGCTGC CGCTCAGCAA GAGCGTCTAC AGGAACACGG
Consensus

Chromatid
Consensus: GACCGCCAAG TGGAACGGCG GGACGGTGTT CGCCTGCATG GCGG

1601-1650

chFST315Fc(3-4)OR: AGGCGCTGCC CATGCGCTTC AGCCAGCGCA CGCTGCAGAA ACAGGCTGGT
chFST315Fc(3-4)SEQ: AGGCGCTGCC CATGCGCTTC AGCCAGCGCA CGCTGCAGAA ACAGGCTGGT
Consensus: AGGCGCTGCC CATGCGCTTC AGCCAGCGCA CGCTGCAGAA ACAGGCTGGT

1651-1651

chFST315Fc(3-4)OR: AAAAAGCTT
chFST315Fc(3-4)SEQ: AAAAAGCTT
Consensus: AAAAAGCTT