MYOSTATIN-LIKE GENE: A POTENTIAL TARGET FOR GENE SILENCING IN GIANT FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII*

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ABSTRACT

Macrobrachium rosenbergii is commonly known as the 'giant freshwater prawn', a commercially important cultivable food species of South and Southeast Asia. Growth retardation and diseases hindering growth are few of the important constrains faced by *Macrobrachium rosenbergii* (*Mr)*, thus highlighting the need of an in-depth study on the growth and growth related genes to improvise this species. Myostatin (MSTN) is an interesting negative growth regulating gene which has been well characterized in vertebrates but studied very little in invertebrates. Thus there is a need to ascertain its functional role more precisely in invertebrates specifically crustaceans. The present study is focused on molecular cloning and characterization of the MSTN gene in *M. rosenbergii* (*Mr*MSTN) followed by gene profiling at different life stages and upon IHHNV infection. It also focuses on further silencing this gene in Giant freshwater prawn, *M. rosenbergii* in order to record any histological changes. In addition, the study also deals with the MSTN gene's influence on other genes of interest related to growth which include myosin heavy chain, dystrophin-dystroglycan complex, tropomyosin, farnesoic acid O methyl transferase, arginine kinase, cyclophilin, and acyl CoA desaturase. We have characterized a full length *Mr*MSTN gene encoding 1619 base pairs (bp) with an open reading frame of 945 bp that encodes 315 amino acid residues. *Mr*MSTN retains all the conserved characteristics which belong to the TGF-β superfamily including the propeptide and mature peptide domain, cysteine residues and a proteolytic cleavage site, RXXR where "XX" denotes Asparagine and Arginine. The bioinformatic analysis confirmed that *Mr*MSTN belongs to the TGF-β superfamily and is evolutionarily conserved especially in the mature peptide domain. *Mr*MSTN gene is ubiquitously expressed in the tested tissues of healthy adults, with the highest expression observed in the muscle. Moreover, the *Mr*MSTN transcripts showed significant ($P < 0.05$) changes at different life stages of

M. rosenbergii as well as in infectious hypodermal and hematopoietic necrosis virus (IHHNV)-challenged prawns. Our histological analysis following the successful *Mr*MSTN dsRNA gene silencing showed the onset of muscle regeneration, thus supporting *Mr*MSTN functional role serving as a negative growth regulator, as similar in vertebrates. Moreover, the *Mr*MSTN also significantly influences the mRNA transcripts of other growth regulating genes, and this activity coincides with a muscle regeneration process that takes place. Our findings, illustrated the role of *Mr*MSTN as a negative growth regulator and its importance in muscle growth and development in *M. rosenbergii*. The *Mr*MSTN could therefore be a potential target for gene manipulation aimed at enhancing the growth and development of *M. rosenbergii*. In addition it could also be developed as a biomarker to address the growth deficiency related problems in *M. rosenbergii.*

ABSTRAK

Macrobrachium rosenbergii yang lebih dikenali sebagai 'Udang Galah', merupakan satu spesies makanan yang berpotensi komersil di Asia Selatan dan Asia Tenggara. Terencat pertumbuhan dan penyakit-penyakit yang menghalang pertumbuhan adalah beberapa kekangan yang dihadapi oleh *Macrobrachium rosenbergii* (*Mr*), sekali gus menonjolkan keperluan sebuah kajian yang mendalam terhadap pertumbuhan dan gen berkaitan pertumbuhan untuk memperbaiki spesies ini. Myostatin (MSTN) adalah pertumbuhan negatif yang menarik yang mengawal selia gen yang telah dicirikan di vertebrata tetapi sangat sedikit dikaji dalam invertebrata. Oleh itu terdapat keperluan untuk menentukan peranannya dengan lebih tepat dalam invertebrata terutamnya krustasea. Kajian kini menumpukan dalam usaha molekul pengklonan dan pencirian gen MSTN dalam *M. rosenbergii* (*Mr*MSTN) diikuti dengan transkripsi mRNA pada peringkat kitaran hidup yang berbeza dan apabila jangkitan IHHNV. Ia juga menumpukan lagi dengan membantutkan gen ini dalam Udang Galah gergasi, *M. rosenbergii* untuk merekodkan sebarang perubahan histologikal. Di samping itu, kajian ini juga berurusan dengan meneliti pengaruh MSTN ke atas gen-gen lain yang berkaitan dengan pertumbuhan yang merangkumi di myosin heavy chain, kompleks dystrophin-dystroglycan, tropomyosin, farnesoic acid o methyl transferase, arginine kinase, cyclophilin, and acyl CoA desaturase. Kami telah berjaya yang memperolehi gen *Mr*MSTN penuh dengan pengekodan 1619 jujukan (bp) yang mempunyai suatu kerangka bacaan terbuka 945 bp yang mengkod bagi 315 jujukan amino asid. *Mr*MSTN mengekalkan semua jem ciri-ciri yang dimiliki oleh Superfamili TGF-β termasuk domain peptide propeptide dan matang, sisa-sisa sisteina dan tapak proteolotik belahan, RXXR di mana "XX" menandakan Asparagine dan Arginine. Bioinformatik analisis yang diperolehi mengesahkan bahawa *Mr*MSTN kepunyaan Superfamili TGF-β dan dipelihara evolusinari terutamanya dalam domain peptide matang. *Mr*MSTN gen didapati dalam semua tisu sihat apabila dewasa

kelak, dengan ungkapan tertinggi yang diperhatikan pada otot. Selain itu, transkrip *Mr*MSTN menunjukkan signifikan ($P < 0.05$) pada peringkat kehidupan berbeza *M. rosenbergii* dan juga selepas udang dicabari dengan virus nekrosis hypodermal dan hematopoietic (IHHNV). Histologikal analisis selepas *Mr*MSTN di nyah-aktifkan dengan berjaya menunjukkan bahawa ia membolehkan pertumbuhan semula otot, sekali gus menyokong bahawa peranannya berfungsi sebagai pengawal pertumbuhan negatif, sama seperti vertebrata. Selain itu, ia juga secara signifikan mempengaruhi transkrip mRNA gen-gen pertumbuhan lain dan aktiviti ini serentak dengan proses pertumbuhan semula otot yang berlaku. Penemuan kami, menggambarkan peranan *Mr*MSTN sebagai pertumbuhan negatif pengawal tumbuhan dan kepentinganya dalam pertumbuhan otot dan pembangunan di *M. rosenbergii*. *Mr*MSTN ini boleh menjadi sasaran yang berpotensi untuk manipulasi gen yang bertujuan untuk meningkatkan pertumbuhan dan pembangunan *M. rosenbergii*. Di samping itu ia dapat juga akan dibangunkan sebagai biomarker untuk menangani kekurangan pertumbuhan berkaitan masalah *M. rosenbergii*.

DEDICATED TO:

BELOVED MOM MDM. PUVANESWARI

AND FAMILY

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

M. rosenbergii	*Macrobrachium rosenbergii*
MSTN	Myostatin
*Mr*MSTN	*Macrobrachium rosenbergii* myostatin
TGF-β	Transforming growth factor-Beta
GDF	Growth and differentiation factor
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
cDNA	Complementary Deoxyribonucleic acid
mRNA	Messenger RNA
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase PCR
qRT PCR	Quantitative real time PCR
RACE	Rapid amplified cDNA Ends
dNTP	Deoxynucleotide
bp	Base pair
aa	Amino acid
Cys	Cysteine
Arg	Arginine
S-S	Suphur-sulphur
BLAST	Basic Local Alignment Tool
SNP	Single nucleotide polymorphic
ORF	Open reading frame
UTR	Untranslated region

NCBI	National Centre for Biotechnology Information
ng	Nano gram
µM	Micro molar
µm	micrometre
OIE	World Organization for Animal Health
IHHNV	Infectious Hypodermal And Hematopoietic Necrosis Virus
PBS	Phosphate buffer Saline
EF-1	elongation factor-1
cT	Cycle threshold
ddCT	Delta delta cycle threshold
ANOVA	Analysis of Variance
SPSS	Statistical Package for the Social Sciences
dsRNA	Double-stranded RNA
ssRNA	Single strand RNA
siRNA	small-interference RNA
GFP	Green fluorescent protein
dsRNA-*Mr*MSTN	Double stranded RNA of *M. rosenbergii* myostatin
dsRNA-GFP	Double stranded RNA of Green fluorescent protein
H&E	Hematoxylin and Eosin
EtOH	Ethyl alcohol
d.p.i	day post injection
MIH	molting inhibiting hormone
MyHC	myosin heavy chain
CHH	crustacean hyperglycemic hormone
DMD	Duchene muscular disease
MF	methyl farnesoate

FaMET	Farnesoic acid O-methyltransferase
AK	Arginine kinase
CypA	Cyclophilin A
SCD-1	Acyl CoA desaturase

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

Aquaculture has been identified as one of the fast growing sectors with a drastic increase in demand for seafood. It is believed to be a promising field for food source which could accommodate the demands of growing population nationwide (De Grave & Ashelby, 2013). The world's aquaculture food source consumption has been predicted to increase along with the sturdy growth in the human population (FAO, 2014). From a production of less than 1 million tonnes per year in the early 1950s, production in 2006 was reported to be 51.7 million tonnes with a value of US$78.8 billion, representing an annual growth rate of nearly 7 % (De Grave & Ashelby, 2013). The aquaculture production in 2011, a contribution worth USD 70 billion has been from approximately 200 culture species comprising of Decapod crustaceans specifically marine species including prawns, lobsters and crabs (FAO, 2012).

The species of interest in the current study, *Macrobrachium rosenbergii* (De Man, 1879) or the commonly known giant freshwater prawn has been a promising source of global seafood production under the freshwater category. *M. rosenbergii* has been ranked as the sixth largest aquaculture species in Asia based on its volume of production (Mather, 2008). For the past fifteen years, *M. rosenbergii* research has been one of the primary focuses in aquaculture studies due to its commercial value (See, Tan, Hassan, Siraj, & Bhassu, 2009). Moreover, this species has been cultivated extensively in its multiple countries of origin, which include Australia and South-East Asia; Bangladesh, Brunei Darussalam, Cambodia, China, India, Indonesia (Jawa), Malaysia, Myanmar, Pakistan, Philippines, Singapore, Sri Lanka Thailand and Vietnam; In addition this species has also been introduced in Japan, China, Israel, Africa, USA and New Zealand (New, 2000).

In Malaysia, the development of larval rearing technique by a FAO expert Shao-Wen Ling, has resulted in a major breakthrough in aquaculture breeding, which further increased the production (Ling, 1969). The giant freshwater prawn, has been widely cultured in several states in Malaysia including Negeri Sembilan, Perak, Kedah, Johor and Sarawak. Though the contribution of *M. rosenbergii* from Malaysia is considered minute compared to the other South-East Asian countries, the production is currently expanding with a high demand along with financial and technological support from the government. Recent sources have reported that *M. rosenbergii* has contributed approximately 24 000 metric tonnes against a target of 23,000 metric tonnes in 2013 and was expected to reach up to 29,000 metric tonnes by 2014 (*ETP Annual Report*, 2013).

Despite the high demand and the potential fast growth of the species, *M. rosenbergii* faces constraints such as being prone to diseases and growth restriction in hatchery based breeding. The group of pathogens affecting *M. rosenbergi* includes viruses, bacteria and fungi. Among the list of diseases those caused by viruses are Infectious hypodermal hematopoietic necrosis virus (IHHNV) infection, white tail disease (WTD), *Macrobrachium* necrosis infection, White spot Syndrome BaculoVirus (WSBV) whereas by bacteria include bacterial necrosis, luminescent larval syndrome and white post-larval disease. On the other hand, fungal infections by fungi such as *Fusarium solani*, *Debaryomyces hansenii* and *Metschnikowia bicuspidata* (Burns, Berrigan, & Henderson, 1979) are yet to be fully characterized. In general, the infections lead to syndromes such as blackening of gills, brown spots, runt deformity syndrome, white muscle, and mortality especially in larvae(Lightner et al., 1992).

Extensive research has been conducted in aquaculture since this industry provides a promising higher income in the short term (Mather, 2008). The major concern

of the research is to increase the production of *M. rosenbergii* in two different aspects that are (i) to increase or improve the growth and (ii) to overcome or minimize the pathogen infection. Thus extensive studies have been conducted through various approaches to find the potential key regulatory genes and markers related to growth, immunity and reproduction (Arockiaraj et al., 2012; Arockiaraj et al., 2011; De Santis et al., 2011; Mohd-Shamsudin et al., 2013; Sagi, Homola, & Laufer, 1991). Research specifically focusing on the growth of *M. rosenbergii* is mostly based on the efficiency in muscle restoration after each molting process, therefore research on key regulatory genes that influence muscle restoration is important to improvise and increase the growth productivity. Thus, the current study deals with looking into a growth related gene, myostatin which controls growth and myogenesis.

Myostatin (MSTN) is one of the important genes that is closely related to the growth of muscle in addition to other genes such as crustacean hyperglycaemic hormones (CHH), actin, tropomyosin, ecdysteroids, fatty acid elongase, crustacean neuropeptide family and molt inhibiting hormone (MIH) (Jung, Lyons, Hurwood, & Mather, 2011b). It is also known as growth differentiation factor-8 and is a member of the sub-family of the transforming growth factor-β (TGF-β) of cytokines. The discovery of this gene in 1997 by McPhersson has led to the wider understanding of skeletal muscle development (Roth & Walsh, 2004), as well as research on muscle disorders such as muscular dystrophy, hyperplasia and hypertrophy (Amali et al., 2008; Chisada et al., 2011; McPherron, Lawler, & Lee, 1997). MSTN plays a vital role in growth as it influences growth related metabolic activity such as myosatellite differentiation, proliferation and muscle regeneration (Rios, Carneiro, Arce, & Devesa, 2002; Wagner, Liu, Chang, & Allen, 2005; Wehling, Cai, & Tidball, 2000). It has been clearly reported that the function of MSTN in vertebrates is as a negative regulator as it inhibits muscle growth and is also

closely associated with the growth developmental stages (Ko et al., 2007; MacLea et al., 2010; McPherron et al., 1997; Shibata et al., 2006).

The discovery of MSTN gene in vertebrates has initiated research of its role in invertebrates. The current status has revealed molecular cloning and characterization of MSTN-like gene from various invertebrates such as the tiger prawn, *Peanus monodon* (De Santis et al., 2011), morotage shrimp, *Pandalopsis japonica* (Kim et al., 2010), fruit fly, *Drosophila melanogaster* (Lo & Frasch, 1999), bay scallop, *Argopecten irradians* (Kim *et al*., 2004), Chinese mitten crab, *Eriocheir sinensis* (Kim, Jeon, & Kim, 2009) , and blackback land crab, *Gecarcinus lateralis* (Covi, Kim, & Mykles, 2008). The MSTN in crustaceans has been initially postulated to serve as a negative growth regulator, similar to that as characterized in vertebrates (Covi et al., 2008; Kim et al., 2009; Kim et al., 2010; MacLea et al., 2010). However, the overall role of MSTN in crustaceans has been interrogated with the converse role of MSTN serving as growth favoring gene as reported in *P. monodon* and *L. vannamei* (De Santis et al., 2011; Qian et al., 2013). Therefore in-depth research is essential to elucidate the MSTN function in invertebrates specifically in crustaceans. Thus, the current study ultimately focuses on isolating and characterizing the gene as well as elucidating its distinct role. The elucidation of *Mr*MSTN role is proposed to be achieved through the mRNA transcripts profiling at various aspects including tissue distribution, different life stages and upon Infectious hypodermal and hematopoietic necrosis virus (IHHNV) infection as well as through gene silencing.

As mentioned earlier, as diseases are also a major concern in the prawn industry, understanding the relationship between growth and diseases, specifically growth affecting infections will be an advantage. In line with that, this study also determine to focus on studying the regulation of MSTN in the presence of a growth affecting virus, IHHNV.

IHHNV has been identified in the early 1980s and has shown infection in penaeid shrimp industry especially in Asia and USA (Tang & Lightner, 2006). Moreover, *M. rosenbergii* specifically the post-larvae and sub-adult stages have been reported to suffer from IHHNV infection which led to a mortality of up to 80% (Hsieh et al., 2006). In addition, IHHNV infection has been reported in wild berried *M. rosenbergii* in Malaysia which is also capable of getting transmitted vertically to the F1 progeny (Hazreen Nita, Kua, Bhassu, & Othman, 2012). The IHHNV infection may lead to Runt deformity syndrome (RDS) in prolonging infection, where the infected prawn suffers from growth deficiencies.

Overall, the present study focuses on identifying and characterizing the MSTN gene for *M. rosenbergii* (*Mr*MSTN) as well as profiling its regulation pattern at different life stages and upon IHNNV challenge. Moreover, in an attempt to obtain a clearer view on the distinct role of MSTN gene, this study also focuses on the dsRNA-*Mr*MSTN gene silencing followed by histological analysis and studies of its influence on other growth regulating genes in *M. rosenbergii.* Thus this study aims to provide a distinct role of the MSTN gene in *M. rosenbergii*, which can help to understand the general role in invertebrates specifically crustaceans. Additionally, this will also give a fundamental idea of the potentiality of the gene in promoting growth for mass production in the future as well as in increasing the aquaculture turnover.

1.1. Objectives

The aim of this study is to identify and characterize a potential growth regulating gene, myostatin and elucidate its possible functional role to in the growth and development of *M. rosenbergii*. The specific objectives of the studies are as follows:-

1. Identification and bioinformatics characterization of myostatin in *M. rosenbergii* :-

   Myostatin for *M. rosenbergii* (*Mr*MSTN) is proposed to be develop from unigenes annotates for MSTN obtained from *M. rosenbergii* transcriptome database followed by bioinformatic characterizations of *Mr*MSTN to confirm the characteristics of *Mr*MSTN.

2. Gene profiling of myostatin for *M. rosenbergii*: Tissue distribution, different life stages and in Infectious hypodermal and hematopoietic necrosis virus (IHHNV) challenged *M. rosenbergii* :-

   Gene expression study is proposed to be performed to quantify the mRNA transcripts of *Mr*MSTN at various stages, thence could aid in elucidating the preliminary role of *Mr*MSTN.

3. *In vivo* dsRNA gene silencing of myostatin in *M. rosenbergii* :-

   (i) Histological observation of phenotypic changes on the muscle

   (ii) Gene expression of growth and development related genes in the transgenic prawn.

   In vivo dsRNA silencing of *Mr*MSTN is proposed to be performed further to elucidate the role of *Mr*MSTN more robustly as well as support previous objective. In addition, this study also interested to profile changes in mRNA

regulation of a cluster of growth related genes as the *Mr*MSTN is being silenced.

1.2. Significance

The cloning and characterization of *Mr*MSTN for *M. rosenbergii* is vital in order to further study the significance of its contribution in development and in growth deficiencies by establishing a correlation with a growth affecting infection, IHHNV. Exploring the newly emerging tool, dsRNA mediated silencing to down-regulate the *Mr*MSTN gene (dsRNA-*Mr*MSTN), enables elucidation of the precise role of *Mr*MSTN in *M. rosenbergii* through subsequent *Mr*MSTN gene expression profiling and histological analysis. Moreover, the gene expression study of other growth related genes in the dsRNA-*Mr*MSTN challenged sample, enables the postulation of a correlation between *Mr*MSTN and other growth related genes as well as the possible roles of *Mr*MSTN.

CHAPTER 2

LITERATURE REVIEW

2.1 *Macrobrachium rosenbergii*

2.1.1 Distribution

Giant freshwater prawn, *Macrobrachium rosenbergii* (Figure 2.1) is the sixth largest aquaculture species in Asia, based on its population (Mather, 2008). It belongs to a large genus of the crustacean class which comprises of about 150 species and is the most favored species for culture (Brown, 1991). *M. rosenbergii* is widely distributed in tropical and subtropical regions of the Indo-Pacific regions including Malaysia, Thailand, Philippines, India, Sri Lanka, Bangladesh, Myanmar, Indonesia and Vietnam (Mather, 2008). It is commonly found in freshwater bodies such as lakes, rivers, swamps, irrigation ditches, canals and ponds (Valenti, Daniels, New, & Correia, 2009).

2.1.2 Taxonomy

M. rosenbergii belongs to the Genus: *Macrobrachium* where 200 species have been documented under this genus. Among all the species reported under this genus, *M. rosenbergii* has gained maximum attention due to its fast growth, large size and meat quality (Jayachandran, 2001). The scientific classification of *M. rosenbergii* is as listed below:

(A)

(B)

Figure 2.1. Giant Freshwater Prawn (*Macrobrachium rosenbergii*). A: Top view and B: side view

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Sub-order: Pleocyemata

Infraorder: Caridea / Natantia

Superfamily: Palaemonoidea

Family: Palaemonida

Subfamily: Palaemoninae

Genus: *Macrobrachium*

Species: *Macrobrachium rosenbergii* (De Man, 1969)

2.1.3 Anatomy

The body of *M. rosenbergii* (Figure 2.2) can be divided into two main sections: the cephalic region and the abdominal region (Hicks & Pierce, 2011). There are five pairs of periopods attached to the head region which are commonly known as the walking legs. The first two pairs of periopods carry a major function as the first pair is used for feeding whereas the second pair has a defending role as well as used for catching food as it is the longest and has more pronounced claws. The head is covered with carapace and has a sharp rostrum with teeth ranging between 12 to 15 at the dorsal part and 8 to 14 teeth at the ventral part (New, 2002). The tail is segmented into six abdominal somites with five pairs of swimming legs or pleopods attached to each segment except for the last segment. The sixth segment carries a tail shaped pleopod which consist of uropods at both sides and a telson in the middle that aids *M. rosenbergii* to move or jerk backwards.

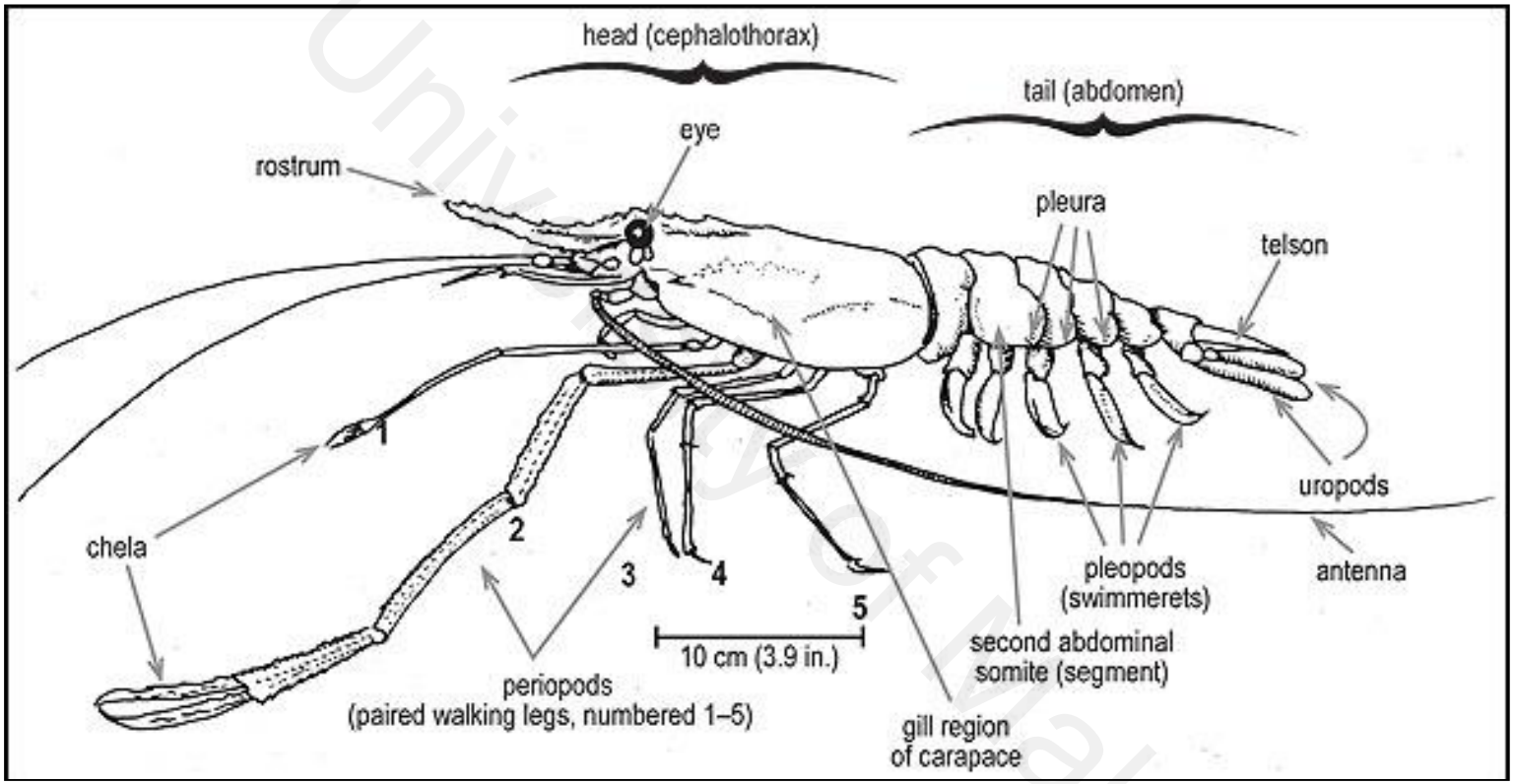


Figure 2.2. External morphology of *Macrobrachium rosenbergii:* The body is mainly divided into two section, head part consisting of eye, rostum, periopods, and gill; Tail part consisting of abdomens, pleura, pleopods, uropods and telson.*

Source: http://extension.missouri.edu/p/G9471

The male and female *M. rosenbergii* can be easily identified as it has distinct physical differences. The male prawn is larger and has a higher growth rate where it can reach up to 320 mm of the total body length compared to the female prawn which can grow up to 250 mm (Holthuis, 1980). In general, the mature male prawn develops a larger cephalothorax and a longer second pair of periopods with more spine compared to the female prawn. Besides that, the structure of the abdominal pleura also aids in differentiating the male prawn from the female prawn as the male has a narrower and shorter abdominal structure compared to a wider and longer abdominal pleura in the female.

The male prawn is classified into two groups based on the size of the claw, either the smaller claw or the larger claw; the prawns with larger claw can be further divided into two groups based on the colors, which are the orange claw and the blue claw (Karplus, Malecha, & Sagi, 2000). The prawn with a smaller claw usually grows slower compared to the prawns with larger claw. A male prawn has the potential to develop from a smaller clawed prawn, to an orange clawed and finally a blue clawed prawn (Ra'anan et al., 1991) wherein the orange claw prawn grows rapidly than the blue clawed males, but the blue claw males shows dominance over other prawns later.

On the other hand, the female prawns exhibit a smaller size with a small head and claw. According to Nandlal & Pickering (2005) the female prawn can also be divided into three types, which are the virgin females (V or VF), berried females (BF) and open brood chamber (spent) females. In a female prawn, the first three abdominal segments are structured in an elongated and wider manner to form a brood chamber to store the fertilized eggs.

2.1.4 Life Cycle

Both freshwater and brackish water are essential to ensure the survival of *M. rosenbergii* and Figure 2.3 illustrates the life cycle of a prawn (Hicks & Pierce, 2011). The female *M. rosenbergii* undergoes several physical changes during the external fertilization process. The sexually mature female undergoes a pre-mating molt and therefore obtains a softer skeleton. Upon mating, the male *M. rosenbergii* will release the sperm into the ventral thoracic region between the periopods of the female *M. rosenbergii* (New, 2002). Subsequently, the fertilization takes place as the eggs are released approximately five to six hours following the deposition of the sperm. The fertilized eggs are stored in the setae of the first four pairs of the pleopods for approximately three weeks (New, 2002). In the early stage, the fertilized eggs appear orange in colour and turn grayish two to three days before hatching. A successful fertilization process may release up to 80, 000 eggs depending on the size of a female prawn (Karplus et al., 2000).

The ready-to-spawn female prawn moves to brackish water or the coastal zone in the river to release the eggs as free swimming larvae (Ling, 1969). The larvae moves in a group and consumes zooplankton as their main food supplement. *M. rosenbergii* larvae undergoes several zoetal stages (Zoea I-XI) before developing into post-larvae (Ling, 1969). According to Nandal and Pickkering (2005) it takes approximately twenty to thirty-five days from the larval stage metamorphosis to the post-larvae stage. Moreover, it undergoes eleven metamorphic stages with distinct changes in its physical trait at each stage (Table 2.1) as described by Nandal and Pickering (2005).

The post-larvae (PL) appears whitish, grey or brownish in colour. During this stage, it also migrates to the low salinity water and adapts to the adults benthic behavior (Ismael & New, 2000). The PL then slowly enters the juvenile stage, which appears similar to PL but is larger in size. At this stage, the rostrum does not have a crest and the teeth are arranged as a compact structure without much space between them. The male prawn with a blue claw conquers the reproduction and the female prawn appears smaller in size compared to the male prawn as it spend more energy for egg production (Nandlal & Pickering, 2005).

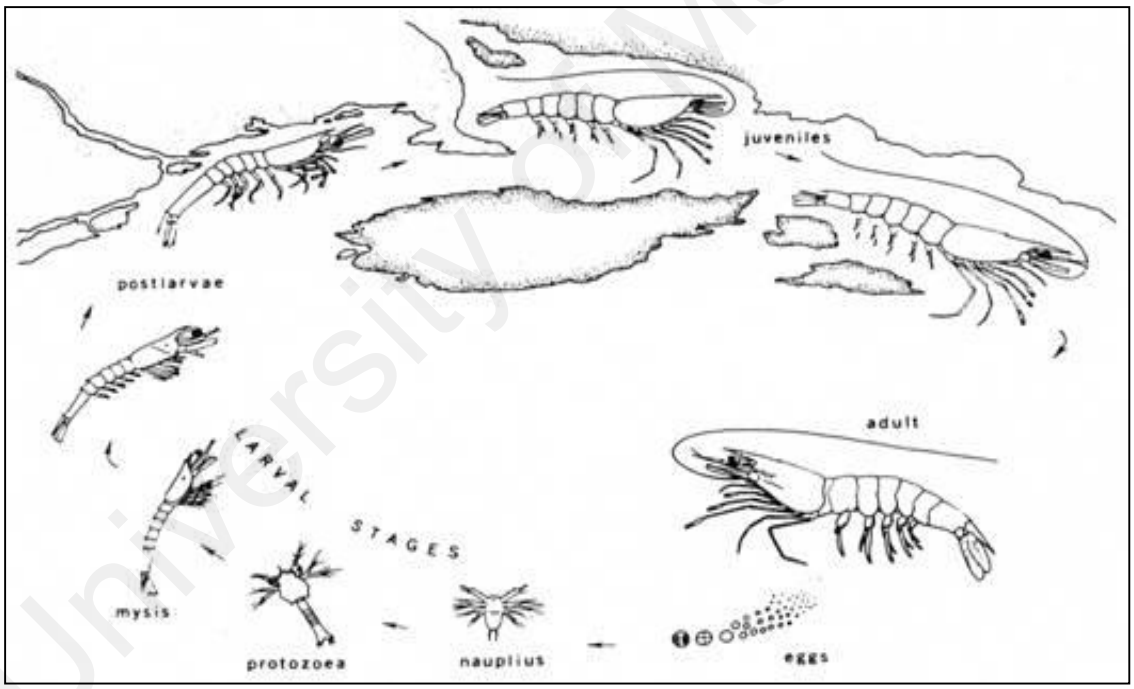


Figure 2.3. Life cycle of *Macrobrachium rosenbergii:* Eggs are released as swimming larvae which undergoes several zoetal stages before entering the post larvae (PL) stage; The PL then move to low salinity water area to develop into juveniles and finally mature adult prawn.*

*** Adapted from,** Hicks & Pierce (1914)
Source: http://extension.missouri.edu/p/G9471

Table 2.1. Table representing keys for identification of larval stages of *Macrobrachium rosenbergii* (Adapted from Nandal & Pickering, 2005)

Larval Stage	Age (Days)	Recognized characters
I	1	Sessile eyes
II	2	Stalked eyes
III	3-4	Uropods present
IV	4-6	2 dorsal teeth
V	5-8	Telson narrows and elongated
VI	7-10	Pleopod buds present
VII	11-17	Pleopods biramous
VIII	13-20	Pleopods with setae
IX	15-22	Endopods of pleopods with appendices internae
X	17-23	3-4 dorsal teeth on rostrum
XI	23-35	Teeth on half of upper dorsal margin

2.1.5 Economical Value of *Macrobrachium rosenbergii*

M. rosenbergii has been the interest of the aquaculture industry for its delicious flesh and high economic demand (Iketani et al., 2011). It remains as an exotic species approximately in forty countries, including the Madagascar (Hanamura, Koizumi, Morioka, Andriantsoa, & Rafalimanana, 2008), Jamaica (Kairo, Ali, Cheesman, Haysom, & Murphy, 2003), United States (Woodley, Slack, Peterson, & Vervaeke, 2002) and Venezuela (Pereira, Eganez, & Monente, 1996). The *M. rosenbergii* has been adapted for an extensive culture following a successful larval rearing techniques (Ling, 1969). It has been reported that the contribution of shrimp industry has touched approximately 3.4 million tons per year worldwide, and Asia has been listed as a major contributor (FAO, 2008). The worldwide annual production of the giant freshwater prawn has begun to surpass 200,000 tons from the year 2002 (FAO, 2008). Understanding the commercial demand of this species, this economically important species subsequently gained much interest among researchers over the period for its genetic improvement and production increment (New, 1995)

2.1.6 Research Background in *Macrobrachium rosenbergii*

In the past twenty years, the research in crustaceans specifically in the growth focuses to improvise the knowledge and productivity of the molt-regulating genes, external factor influencing growth, growth developments and age limitations (Hartnoll, 2001). More specifically, the *M. rosenbergii* has been extensively studied widely in multi-disciplinary aspects including breeding, feeding and nutrition, immune, reproduction and growth. The ultimate objective of all these studies has been to establish an improved brood stock of the *M. rosenbergii* with an increased productivity that could translate to a higher revenue.

The first breakthrough in *M. rosenbergii* breeding made by Ling (1961) and the second breakthrough into a successful commercial-scale hatchery production of post-larvae by Fujimura and Okamata (1972) have further led to successful larval rearing and many other breeding and growth related programs. Following that, advances in technology enabled the characterization of many growth regulating, immune, fat metabolism and reproduction related genes (Arockiaraj et al., 2011; Jung et al., 2011b; Piyaviriyakul & Darawiro, 2014). Additionally, rapid detection kits are also readily available in the market to identify pathogenic infections that affect the growth production in the early stages of *M. rosenbergii* culture system, thus helping the control of diseases. These infections include those caused by White spot syndrome virus (WSSV), Infectious hypodermal and hematopoietic necrosis virus (IHHNV), Nodavirus and White tail disease (WTD) (Lightner & Redman, 1998).

Looking more specifically into the growth related genes, there are a series of genes related to growth and development that have been characterized for *M. rosenbergii* including actin (Zhu, Dai, Liu, & Yang, 2005), crustacean hyperglycemic hormone (Chen, Lin, & Kuo, 2004; Sithigorngul, Jaideechoey, Saraithongkum, Longyant, & Sithigorngul, 1999), molt inhibiting hormone (MIH) (Allayie et al.,2010) and vitellogenesis inhibiting hormone (VIH) (Okuno et al., 2002). Moreover, recent advances in the next-generation sequencing (NGS) has driven the possibility of the transcriptomic analysis of *M. rosenbergii* and subsequent identification of an extensive log of expressed genes that influences growth and development (Jung et al., 2011; Mohd-Shamsudin et al., 2013).

The higher demand in research for the improvement of *M. rosenbergii* has prompted the current study to focus on a growth regulating gene, myostatin which belongs

to the transforming growth factor - β (TGF-β) family. This gene is known to bear unique characteristics in vertebrates but remain unclear in invertebrates (De Santis et al., 2011; Kim et al., 2009; Kim et al., 2010; MacLea et al., 2010; Qian et al., 2013).

2.2. TRANSFORMING GROWTH FACTOR–B (TGF- B) AND MYOSTATIN

The transforming growth factor-β or the tumor growth factor plays a major role in the alteration of the gene expression particularly in regulating embryonic development as well as maintaining tissue homeostasis in adult animals (McPherron & Lee, 1997). There are approximately more than 50 ligands that have been characterized for TGF-β superfamily. In addition to this it has been classified into three major sub-families including bone morphogenic protein (BMP), TGF-β and activin (Kollias & McDermott, 2008). BMP has been involved in the growth and development during mammalian embryogenesis whereas TGF-β and activin are involved in the later part of embryogenesis as well as in organ development (Massague & Chen, 2000).

TGF-β, is a superfamily of cytokines with 3 isoforms labeled TGF-β1, TGF-β2 and TGF-β3 (Munger et al., 1997). TGF-β is responsible for the growth and development as it is involved in cell proliferation and development, maintaining homeostasis of the cell and also apoptosis (Massague & Chen, 2000). TGF-β has two big domains namely the propeptide domain or commonly known as latency associated protein (LAP) attached to another mature peptide domain with a di sulfide bond (Patel & Amthor, 2005).

In general, the TGF-β family members share similar signalling pathways (Figure 2.4). Initially, the gene gets activated as it binds to a type II serine/threonine kinase transmembrane receptor. It then binds to a type I receptor within the extracellular cell. Subsequently, the activated receptor molecule complex activates SMAD cascade reaction

in the cytoplasm to form a rigid complex which then migrates to the nucleus. A group of cofactors and transcription factors contributes in developing a higher complex from the earlier SMAD complex that regulates the transcription of the target gene (Kollias & McDermott, 2008). Looking more specifically into the TGF-β1 family, myostatin (MSTN) has been the gene of interest in this study. Thus, this gene has been reviewed in brief of its biochemical pathway here and has been detailed along the chapters with the various aspects including the extensive research in the vertebrate and invertebrates; role in the growth and growth affecting diseases; gene silencing and histological analysis; and influence on other growth regulating genes.

MSTN is also known as a growth differentiation factor-8 (GDF-8). In similarity with the characteristic feature of TGF-β family, the MSTN exists in abundance in an inactive form as the mature peptide remains attached with the latency associated propeptide. The active MSTN is produced as the mature peptide gets cleaved at the conserved proteolytic cleavage site, RXXR (Kim et al., 2010). Moreover, the mature peptide possesses the conserved cysteine residues which are involved in the inter-chain and intra-chain di-sulphide (S-S) bondings.

As mentioned earlier, the MSTN shares a similar signaling TGF-β pathway (Figure 2.5). The activated MSTN initiates the activation of activin membrane receptors. This activin type II receptor is followed by phosphorylation and activation of the activin type I receptor. The activin complex then initiates a cascade of signaling SMAD transcription factors (Kollias & McDermott, 2008; MacLea et al., 2010). The rigid complex of SMAD that forms the final part then regulates the transcription of MSTN (Patel & Amthor, 2005). The MSTN gene expression is inhibited in the presence of

ligands which include the follistatin, follistatin related genes (FLRG), propeptide and GDF-associated serum proteins (GASP-1) (Patel & Amthor, 2005).

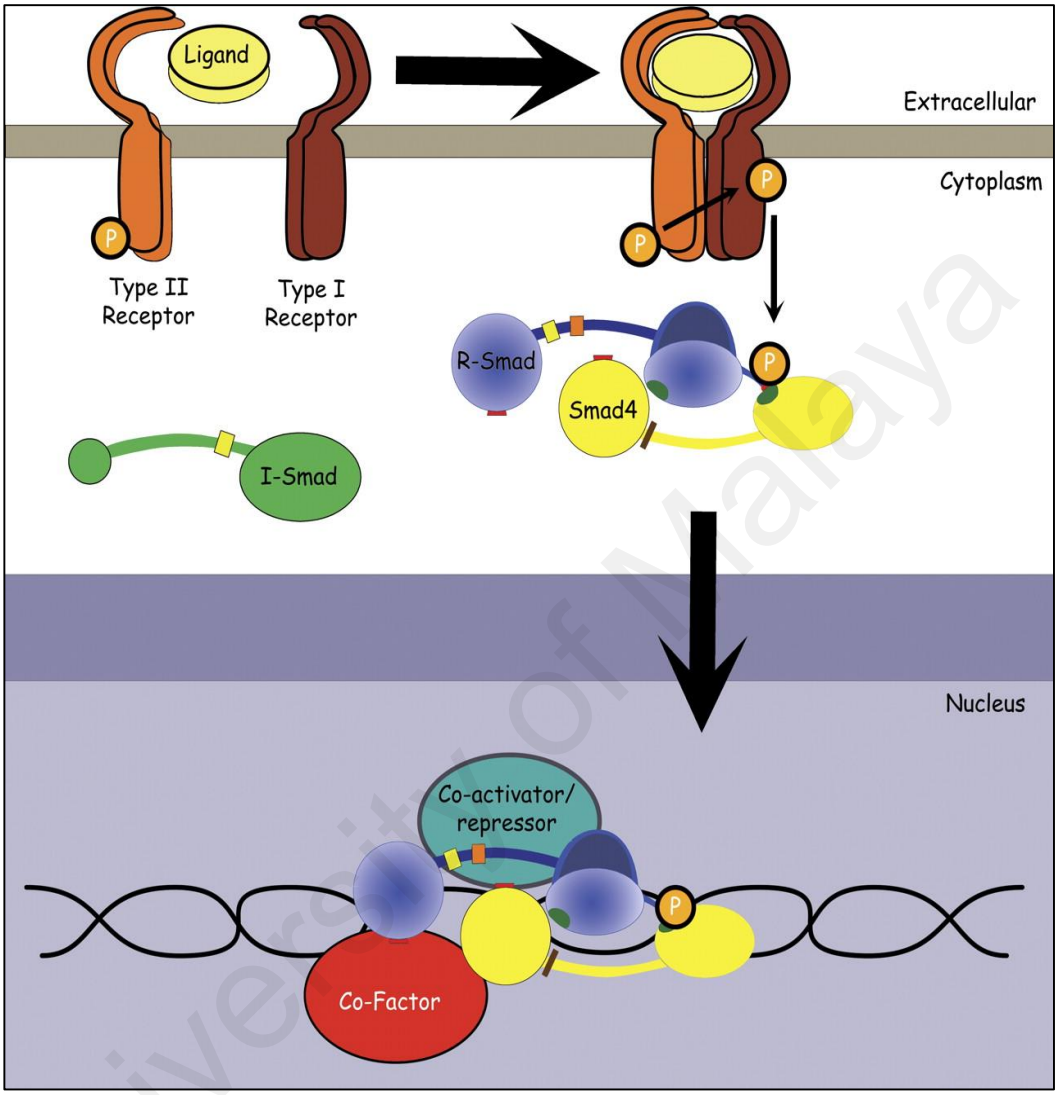


Figure 2.4. The signalling pathway of TGF-β - The TGF superfamily member (ligand) binds to the type II in which gets activated, transphosphorylate and activates the type I receptor. Then, the type I receptor phosphorylates and initiate a cascade of Smads reaction. The resulting Smads reaction complex translocates into the nucleus to regulate target gene transcriptions.

*Adapted from Kollias & McDermott, 2008

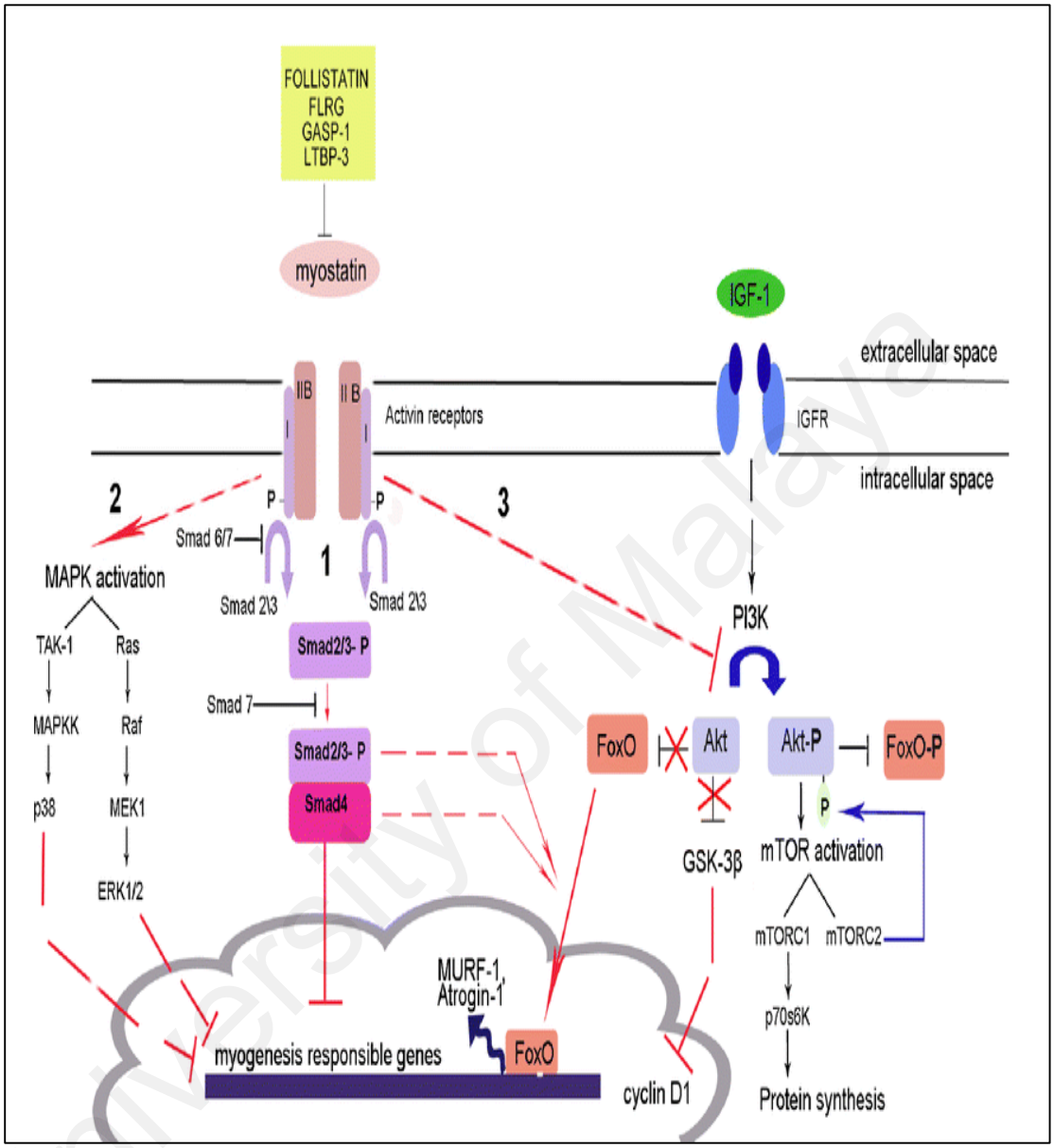


Figure 2.5. Schematic representation of myostatin (MSTN) signaling through TGF-β pathway: The MSTN binds to the type II in which gets activated, transphosphorylate and activates the type I receptor. Then, the type I receptor phosphorylates and initiate a cascade of Smads reaction. The resulting Smads reaction complex translocates into the nucleus to regulate target gene transcriptions of MSTN.

*Adapted Elkina et al, 2011

2.3 RNA INTERFERENCE (RNAi)

RNA interference (RNAi) is a well-established tool used in silencing a gene. RNAi approach was first introduced in *Caenorhabditis elegans* where the par-1 gene was silenced through antisense RNA administration to study its functional role (Guo & Kemphues, 1995; Tabara, Grishok, & Mello, 1998). Since then, gene silencing has been a useful and an effective method to understand the functional role or the pathway of a gene. The gene silencing is made possible through the delivery of molecules including small-interference RNA (siRNA), small-hairpin RNA (shRNA) and double-strand RNA (dsRNA).

Double-stranded RNA (dsRNA) mediated silencing is the technique involving the disruption of protein expression at the post-transcriptional level (Acosta, Carpio, Borroto, Gonzalez, & Estrada, 2005; Montgomery & Fire, 1998). Figure 2.6 shows the gene silencing pathway by a dsRNA molecule. The dsRNA molecule is cleaved to short-interfering RNA (siRNA) with 21-28 nucleotides by RNAse III like enzyme called dicer in the cytoplasm. It then binds to an agronaute component in the RNA- induced silencing complex (RISC) (Meister & Tuschl, 2004). The RISC then gets activated and unwinds the double stranded siRNA to a single stranded siRNA which then binds to a complementary target gene of mRNA and interferes with the protein translation (Franca et al., 2010). The dsRNA injection has been established as more productive in gene knockdown studies in crustaceans (Sagi, Manor, & Ventura, 2013). Earlier dsRNA silencing of *mex-3* gene in *C. elegans* showed that the RNAi effect is seen in subsequent F1-progeny (Fire et al., 1998).

MSTN has been silenced in mice primarily to elucidate its functional role (McPherron et al., 1997). In addition MSTN gene silencing also aid in determining its role and influence in the growth and muscle affecting diseases including mdx mice (Girgenrath, Song, & Whittemore, 2005; Hu et al., 2013; Jain, Singh, Kadam, & Sarkhel, 2010; Malerba et al., 2012).

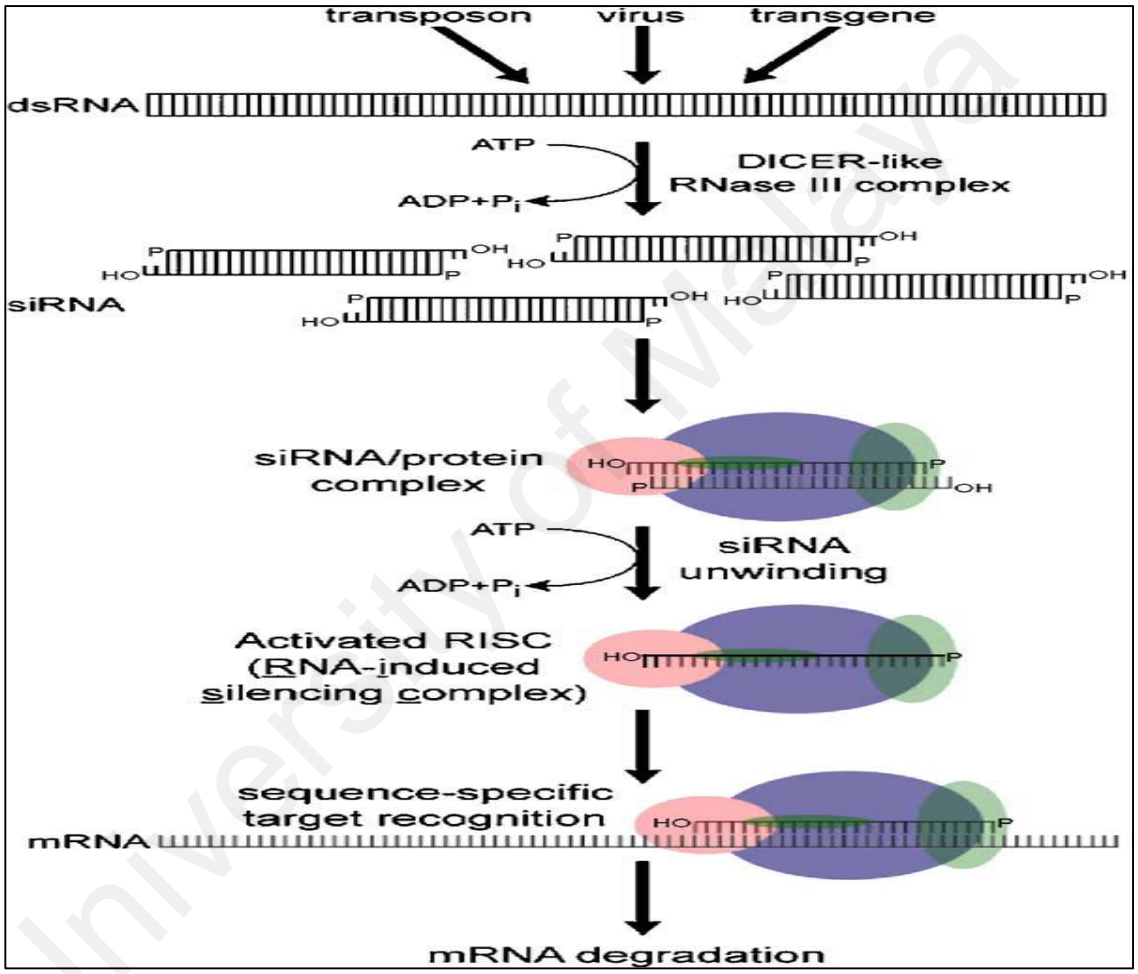


Figure 2.6. The dsRNA mediated gene silencing mechanism: The inserted dsRNA molecule is cleaved into siRNA by dicer-like RNAse III complex. The siRNA then binds to RISC complex, get activated and unwind to retain antisense strand and degrade the sense strand. The RISC complex then binds to the complementary sequence of targeted mRNA and resulting on its degradation*

*Adapted from Roth et al, 2004

2.4 MYOSTATIN (MSTN)

2.4.1 Myostatin Role in Myogenesis and Muscle Hypertrophy

The available insights into MSTN are closely associated with the growth, thus it is necessary to understand the myogenesis process which involves the muscle development in order to understand the MSTN function (Weiner, 2009). The initial muscle fiber is formed from mulitipotent cells which originate from the mesoderm and subsequently differentiate into mature muscle cells called the myoblast (Weiner, 2009). Transcription factors such as MyoD and Myf5 initiate the differentiation by activating muscle-specific genes (Weiner, 2009). The myoblast then proliferates until it leaves the cell cycle and fuses to form multi-nucleated myotubes (Joulia et al., 2003). Finally it develops into a complete skeletal muscle tissue, the contractile unit in the presence of specific proteins.

MSTN is well characterized as a negative growth regulator during the myogenesis process in vertebrates (Rios, Carneiro, Arce, & Devesa, 2001; Tsao et al., 2013). The absence of MSTN is known to result in the proliferation of C_2C_{12} and Bovine Primary Myoblast (Langley et al., 2002). Figure 2.7 shows a model of MSTN signaling that is exerted in the inhibition of myoblast proliferation and myocytes differentiation respectively. In vertebrates, MSTN in the adult organism, is known to restrain the activation and differentiation of the satellite cells, a key point which plays an essential role in the muscle regeneration (Bischoff, 1994; McCroskery, Thomas, Maxwell, Sharma, & Kambadur, 2003).

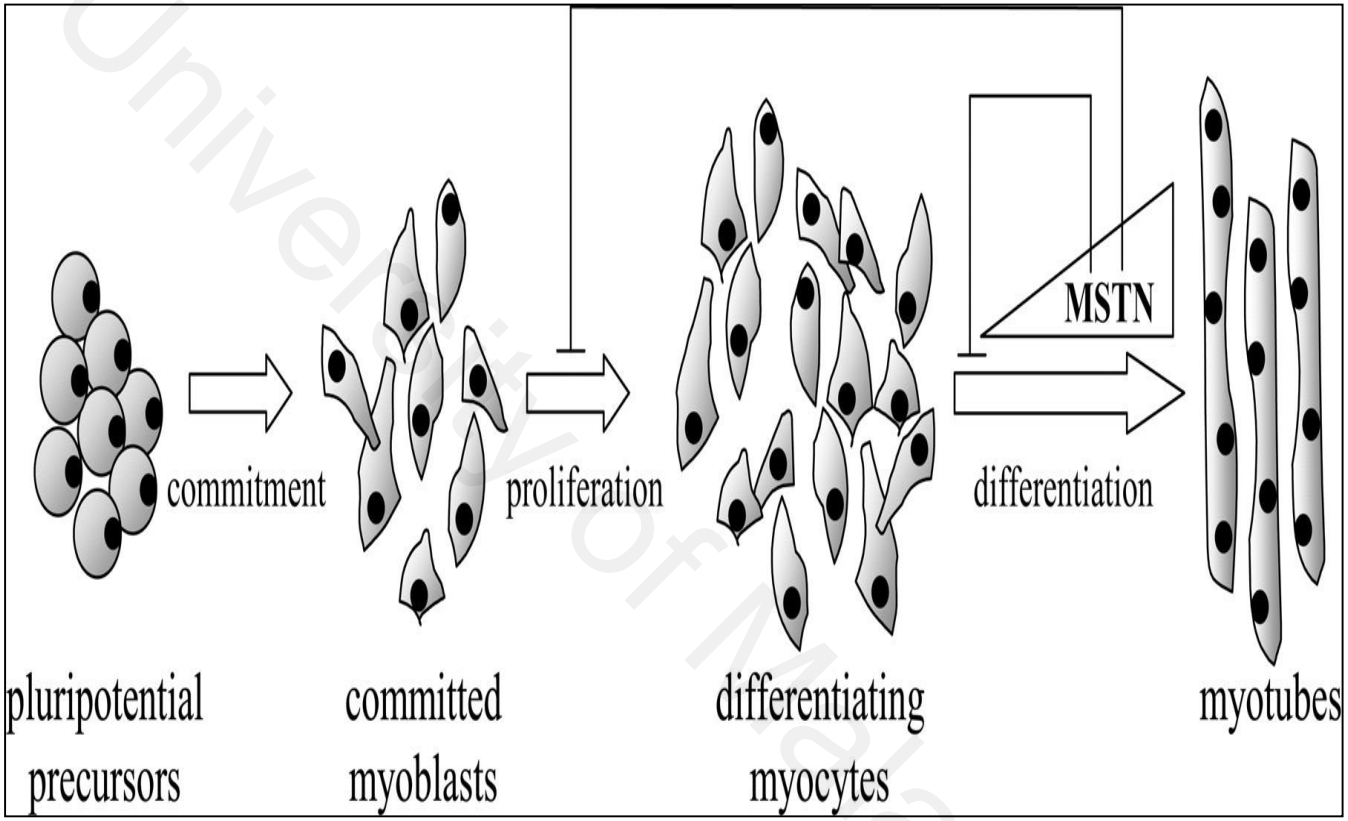


Figure 2.7. Model illustrating the role of MSTN in myogenesis. In the presence of MSTN signal both the committed myoblast proliferation and myocyte differentiation is inhibited*

*Adapted from: Rios et al., 2002

Disruption in the function of MSTN either by natural mutation or MSTN gene knockout will promote an increase in muscle mass either by hyperplasia or hypertrophy or both (Acosta et al., 2005; Nishi et al., 2002; Tripathi, Ramani, Patel, Rank, & Joshi, 2013). Hyperplasia involves an increase in the number of muscle fibers, whereas hypertrophy contributes to an increase in the number and size of existing muscle fibers. Such doubling of muscle phenomena has been earlier observed in the gene knockout of MSTN in mice by McPhersson and Lee (1997) and in the cattle of the Belgian Blue Cattle Breed (BCBB) due to a natural eleven base pair deletion by Grobet et al (1997). The first MSTN deficiency case was recorded in humans by Schuelke et al (2004) where a 4 year old German boy showed an astonishing muscle growth since birth. In all the cases, the muscles were known to primarily undergo muscle hypertrophy while hyperplasia to a lesser extent. Hypertrophy and hyperplasia are closely related to satellite cells as these cells contribute significantly to the respective event, in addition being influenced by growth regulating genes (Hawke & Garry, 2001).

2.4.2 Satellite Cells

Skeletal muscle satellite cells are quiescent cells that remain dormant between the sarcolemma and basement membrane of terminally differentiated muscle fibers (Mauro, 1961). The satellite cells can be distinguished from the myonuclei which contains most of the cell volume. Figure 2.8 shows the satellite cell position in adult mice during the activated stage and during the dormant stage (Hawke & Garry, 2001). The satellite cells are referred to as muscle stem cells and are capable of self-copying into larger number of satellite cells (Schultz & McCormick, 1994). It is vital for the muscle regeneration and Figure 2.9 shows the satellite cell cycle from an injured cell during muscle regeneration (Hawke & Garry, 2001).

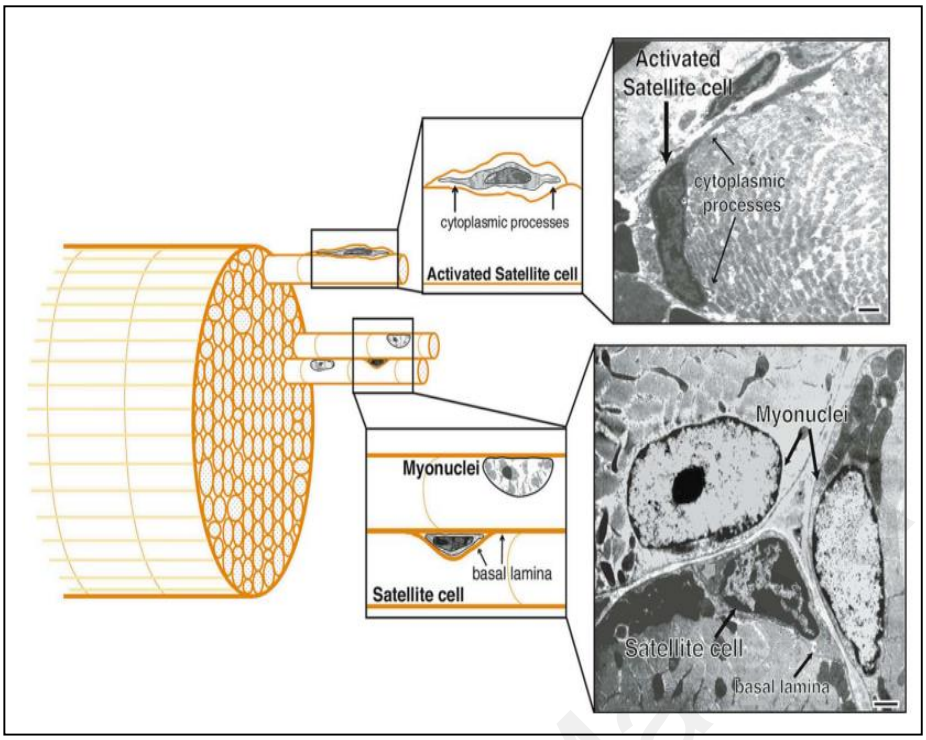


Figure 2.8. Position of the activated satellite cell in an adult injured muscle and at the dormant stage in a normal tissue.*

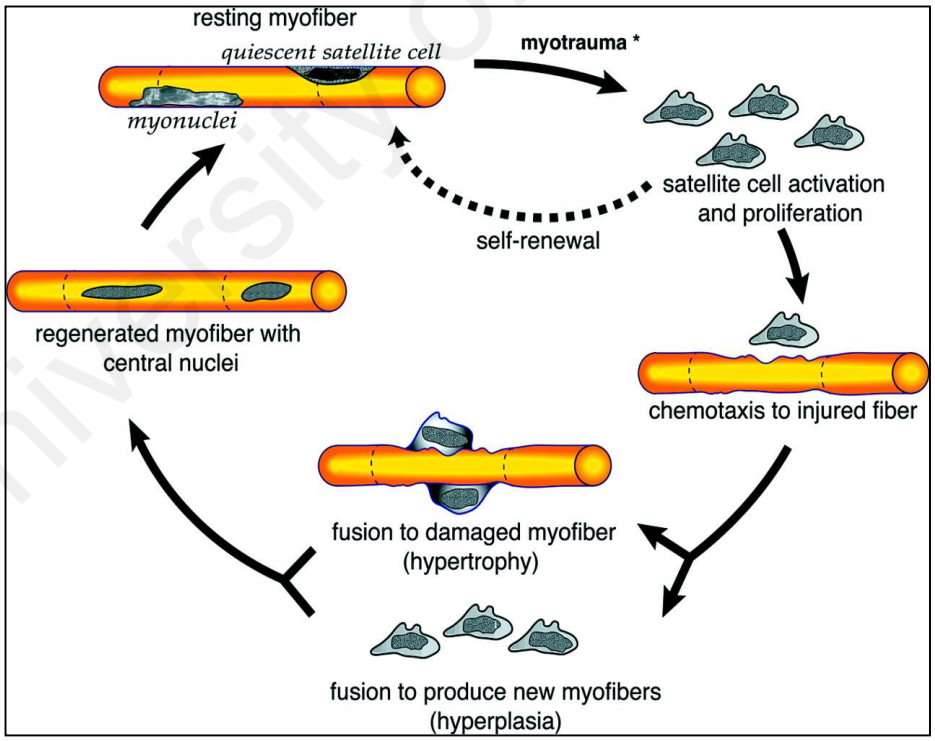


Figure 2.9. Process of satellite cell response to myotrauma: The quiescent satellite cell gets activated and proliferate in response to any mechanical injury or tension created. Some of the satellite cells proceed to self-renewal and some migrate to fused with the myofiber at the injured area to generate new myofibers. The myofiber with centrally located nuclei indicates muscle regeneration. *

*Adapted from Hawke and Garry, 2001

MSTN satellite cell activation in the myogenesis process is followed by hypertrophy and has been proven in many *in vitro* and *in vivo* studies (Cooper et al., 1999; Steelman, Recknor, Nettleton, & Reecy, 2006). In the event of muscle hypertrophy, satellite cell functions to increase the size and number of contractile proteins in muscle fibers. Moreover, the number of satellite cells are abundant in a muscle fiber which is influenced by the varied types of muscle; In general satellite cells are seen greater in type-I or slow-twitch oxidative fibers compared to type-II in the absence of MSTN (Allen & Unterman, 2007; Carlson, Booth, & Gordon, 1999).

2.4.3. Myostatin Role in Growth Affecting Disease

MSTN has been identified to have therapeutically potential in conjunction with its unique characteristics as a negative growth regulator. The gene was further exploited for treating disease affecting muscle mass including cancer, HIV/AIDS, sarcopenia, muscle atrophy, and Duchenne muscular dystrophy (DMD) in which all the cases lead to muscle loss or wasting (Malerba et al., 2012). The MSTN gene disruption in an mdx mouse, has been proven experimentally to onset the muscular hypertrophy (Patel & Amthor, 2005). Moreover, *in vivo* research has also provided evidence that MSTN inhibition aids in bone formation along with increase in the muscle mass (Hamrick et al., 2010). On the other hand, the elevated expression of MSTN was observed in patients that exhibits cachexia, a syndrome that leads to reduction in a weight (Zimmers et al., 2002). Gleaning the role of MSTN in diseases, this study intends to profile the MSTN regulation in the presence of a specific growth affecting disease that affects *M. rosenbergii*. This has been elaborately discussed in Chapter 4.

2.4.4 Histological Study of Myostatin

In vertebrates, the MSTN gene has been silenced and further microscopic observations have been analyzed to perceive changes in the muscle tissue including satellites cell activation, fiber sizes and adipocyte presence in comparison to the control. The most common staining method used in the histological study was Hematoxylin and Eosin (H&E) staining followed by immunohistochemistry including DAPI staining. Harry's H&E staining is a potent fixative displaying nuclear, cytoplasm and extracellular matrix features (Avwioro, 2011). The ultimate principal of the staining chemistry rely on the acid-base affinities between the stain and the tissue. In this consecutive staining of H&E, the hematoxylin with a deep blue-purple colour is responsible to stains nucleic acid whereas eosin with pink colour stains cytoplasm and proteins non-specifically (Fischer, Jacobson, Rose, & Zeller, 2008).

There have been distinct reports of the histological changes in the tissues, in conjunction with the disruption or up-regulation of MSTN from the *in vitro* and *in vivo* studies in vertebrates (Chisada et al., 2011; Cooper et al., 1999; Jain et al., 2010). In addition, the histological analysis of the tissue with disrupted MSTN in Medaka (Japanese rice fish) has shown an increase in the fiber size specifically at the adult stage (Chisada et al., 2011). The H&E staining and microscopic observation of an artificially injured muscle fiber in Figure 2.10 (Hawke & Garry., 2001) provides a clear histological observation of a progressive event in a muscle regeneration which can be used as a reference in histological analysis in later part of the study. The generation of new fibers are indicated with the presence of centrally located nuclei fibers as well as the fusion of multiple satellite cells (Hawke & Garry, 2001).

However, there is no such histological evidence which has been documented in invertebrates, specifically crustaceans. The available information of MSTN in crustaceans is to the extent of gene expression (Covi et al., 2008; Kim et al., 2009; Kim et al., 2010; MacLea et al., 2010) and alteration in body weight upon gene disruption (De Santis et al., 2011; Lee et al., 2015). Thus this study will be reporting preliminary histological changes following the disruption of MSTN gene in *M. rosenbergii*. This will be very useful in aiding the elucidation of the functional role of *Mr*MSTN efficiently.

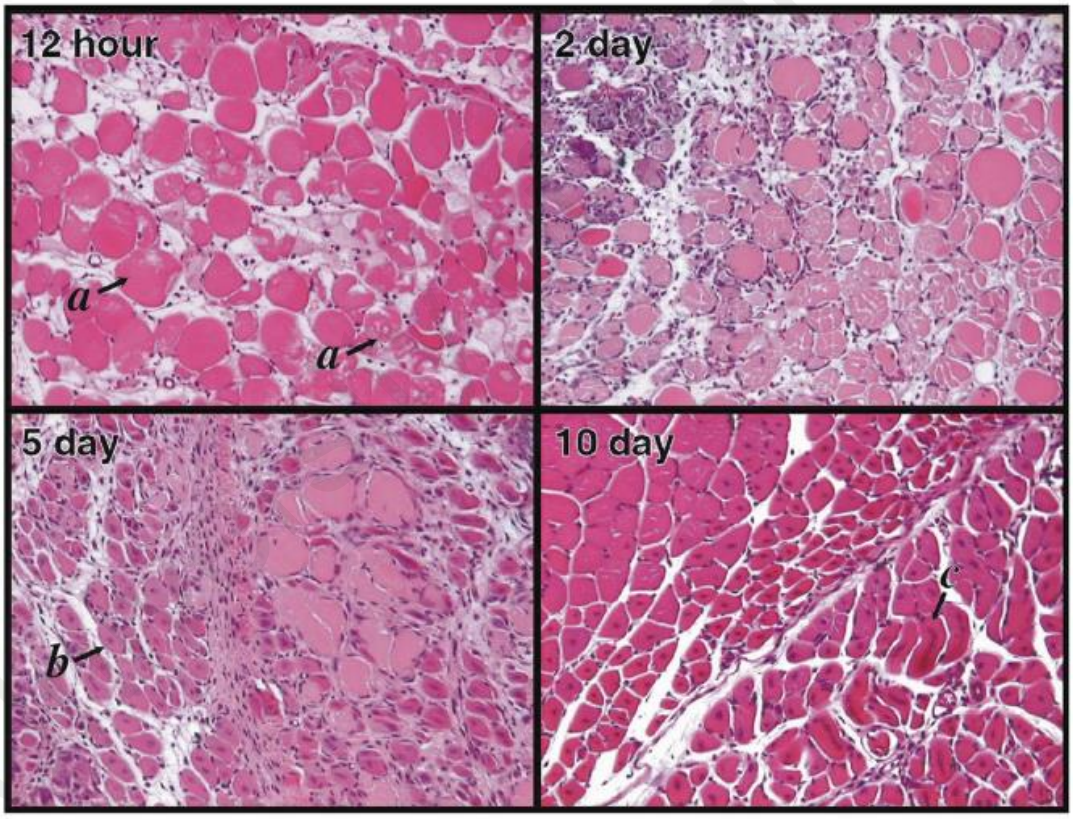


Figure 2.10. Transverse section of the adult gastrocnemius muscle stained with H&E following response to toxin- induced injury at defined intervals including 12 hour, 2 day, 5 day and 10 day. The b denotes fiber with central nuclei and c denotes fusion of multiple nuclei. Bar 100 mm.

*Adapted from: Hawke & Garry., 2001

2.4.5 Correlation of Myostatin with Other Putative Growth and Muscle Development Related Functional Genes

Crustaceans are known to form a large diverse group in the invertebrates. Though their contribution in the aquaculture sector has gained major concern, the study related to the growth improvement particularly the physiology and biochemistry of their skeletal muscle is still lacking (Koyama et al., 2012). There are lists of growth regulating genes which have been identified in crustaceans including the myosin heavy chain, molting inhibiting hormone (MIH), vitellogenesis or gonad-inhibiting hormone (VIH/GIH), crustacean hyperglycemic hormone (CHH) and the tropomyosin (Chang, 2001; El Haj, 1996; Jung et al., 2011b). Although, MSTN has been well characterized in vertebrates and has begun to be explored in invertebrates, its correlation with other growth related genes still remains unclear in both the groups.

To date, only few studies have been conducted to elucidate the influence of MSTN on other growth related genes in vertebrates and is yet to be explored in invertebrates. The MSTN knockout in wild type mice shows an alteration in the expression of myosin heavy chain (MHC) isoforms where the faster MHC isoforms express greater than the slower isoforms (Girgenrath et al., 2005). Similar type of alterations in the gene expression of fast and slow MHC have also been observed in a five week old pectoralis muscle of a null MSTN mice (Steelman et al., 2006). Additionally, the MSTN knock-out study has also proven to influence the fat metabolism by affecting the adipose tissue mass (McPherron & Lee, 2002). Moreover, the up-regulation of Growth Associated Serum Protein -1 (GASP-1) mRNA expression in an attempt to elucidate the PPAR-β gene functional role in myogenesis has also shown to down-regulate the MSTN mRNA transcripts indirectly (Bonala et al., 2012). Besides that, the

MSTN has been postulated to carry a diverse functional role in accordance with a ubiquitous expression in both muscle and non-muscle tissues as reported in vertebrates and invertebrates (Kim et al., 2010; Ostbye et al., 2001).

Thus, this has provoked interest in the current study to investigate the downstream impact of a silenced MSTN gene on other growth regulating and muscle development genes. Few potential candidate genes of interest for the growth and muscle development have been selected based on literature which includes myosin heavy chain, tropomyosin, dystrophin-dystroglycan complex, farnesoic acid O methyl transferase, cyclophilin, and acyl co-A desaturase (Gabriel, 2009; Jung et al., 2011b). The selected genes initially belong to other functional groups including growth, reproduction, fat metabolism and immunity. The arginine kinase was included along as for its role to regulates energy metabolism specifically in extreme condition, expressed high in muscle of *M. rosenbergii* and response to similar pathogenic infection (IHHNV) as MSTN (Arockiaraj et al., 2011). The review for each gene selected is as explained below.

Table 2.2. Genes of interest related to growth and muscle development in *Macrobrachium rosenbergii.*

Group	**Gene**
Growth Regulation	Myosin heavy chain Tropomyosin Dystrophin-dystroglycan complex
Reproduction	Farnesoic Acid O-Methyl Transferase
Immunity	Arginine Kinase Cyclophilin
Fat Metabolism	Acyl Co A Desaturase

2.4.5.1 Myosin Heavy Chain (MyHC)

Myosin is made up of two heavy chains and four light chains and plays a vital role in muscle contraction (Harrington & Rodgers, 1984). It is an ATP-dependant motor protein with many isoforms where its expression is crucial for muscle movement and myofibril stability (Wells, Edwards, & Bernstein, 1996). To date, thirteen myosin genes have been characterized for the myosin family through genomic analysis where myosin I and II are being studied extensively and known to occur most abundantly in eukaryotes (Lodish, Berk, & Zipursky, 2000). Myosin II influences the muscle contraction and cytokinesis, whereas myosins I and V are related to cytoskeleton-membrane interactions such as the transport of membrane vesicles (Lodish et al., 2000). In most cases, myosin and ATPase collide with actin which results in cellular movement and this process depends on the hydrolysis of ATP which supports the drive of the cellular movement (Gauvry, Mohan-Ram, Ettelaie, Ennion, & Goldspink, 1997; Mermall, Post, & Mooseker, 1998; Sellers, 2000).

The first MyHC for a crustacean was characterized from a deep abdominal flexor muscle of the lobster, *Homarus americanus* (Cotton & Mykles, 1993). Following that, MyHC has been characterized in crayfish, *Procambarus clarkii* (LaFramboise et al., 2000), Atlantic pink shrimp, *Farfantepenaeus paulensis* (Kamimura et al., 2008), kuruma shrimp, *Marsupenaeus japonicas* (Koyama et al., 2012), black tiger prawn, *Penaeus monodon* and Pacific white shrimp, *Penaeus vannamei* (Koyama, Akolkar, Piyapattanakorn, & Watabe, 2012b).

MyHC has been considered to be an important gene with a potentiality to serve as a marker during a transition period in muscle regeneration (Yan et al., 2003). MyHC

has been reported to have many isoforms including the embryonic and neonatal MyHC followed by adult fast and slow MyHC (Esser, Gunning, & Hardeman, 1993; Sartore, Gorza, & Schiaffino, 1982; Whalen, Harris, Butler-Browne, & Sesodia, 1990). Each isoform has been reported to have different expression patterns, as influenced by different types of fibers in the skeletal muscle (Medler & Mykles, 2003). Thus, the differential expression of MyHC isoforms is very useful in identifying the characteristics features of a skeletal muscle based on the physiological, histochemical and the protein abundance in the muscle (LaFramboise et al., 2000). Moreover, the different isoforms of MyHC also aids to distinguish the role of nerves in the regulation of muscle regeneration (Musaro, 2014).

2.4.5.2 Tropomyosin

Tropomyosin is a double-stranded alpha helical coiled protein and a member of thin filament. The Figure 2.11 shows an interaction between tropomyosin and myosin which leads to a smooth muscle contraction (Marston & El-Mezgueldi, 2008). Besides that it also serves as an actin stabilizer (Yu & Ono, 2006). These two features of a tropomyosin shows its importance in the muscle contraction event and studies have reported that both the events are independent of each other (Perry, 2008). To date, three isoforms have been characterized for tropomyosin including the TnI, TnT and TnC which are tissue specific and exist abundantly in the smooth muscle (Zot & Potter, 1987).

Tropomyosin has been reported to interact with myogenic factors including myocyte-specific enhancer-binding 2 (MEF2) which influences the high level of muscle expression (Lin, Bour, Abmayr, & Storti, 1997). MEF2 is a transcription factors that involve in the development of skeletal, smooth and cardiac muscle (Baker et al., 2005)

and also a key regulator of myogenesis in vertebrates (Lin, Nguyen, Dybala, & Storti, 1996). Thus, the current study is focused on observing any changes in the regulation of tropomyosin specifically targeting the changes in mRNA regulation within the MEF2 region in tropomyosin.

In crustaceans, supporting elements including tropomyosin are reported to regulate muscle contraction as the expression of genes encoding fast and slow myosin isoforms are elevated (Abdel Rahman, Kamath, Lopata, & Helleur, 2010; Medler, Lilley, & Mykles, 2004; Medler & Mykles, 2003). Besides that, an alteration in the tropomyosin mRNA transcripts has also been observed in the presence of diseases such as tumor growth, myopathies and also allergy (Fernandes et al., 2003; Lam et al., 2012). Moreover, tropomyosin is also reported to serve as a major allergen especially in crustaceans, mollusks and also insects including dust mites and cockroaches. The allergy is mainly caused as the topomyosin binds to IgE, where there are five different binding affinities available for IgE to bind within the crustacean tropomyosin (Lehrer, Ayuso, & Reese, 2003).

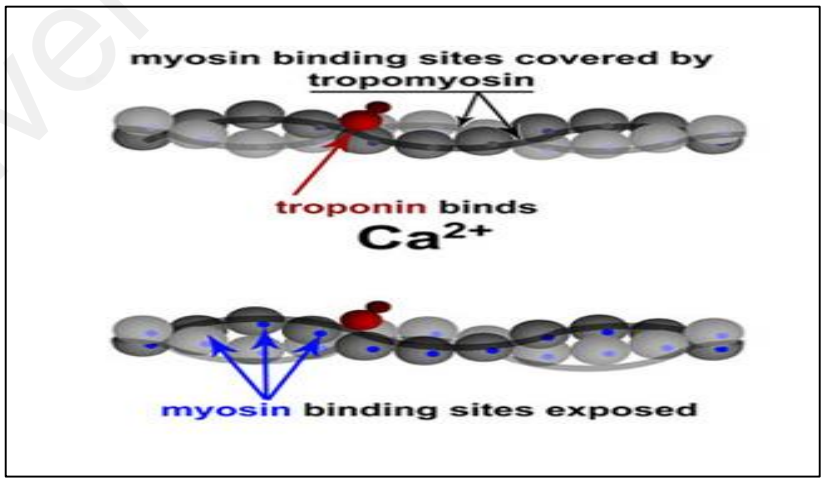


Figure 2.11. Illustration showing the interaction between actin with tropomyosin and troponin (red). As the troponin binds to Calcium (Ca^{2+}) the tropomyosin is released from the myosin-binding sites thus exposing it. *

*Modified from Lehman et al. (1994)

2.4.5.3 Dystrophin – Dystroglycan

Dystrophin or more commonly known as the DMD gene, is the largest known gene with approximately 79 exons encoding a full length 14 Kb mRNA transcript and protein with a molecular weight of 427 kDa (Koenig et al., 1987). It belongs to the β-spectrin/α-actinin proteins family thus has the conserved characteristics constituting an NH2-terminal actin binding domain and multiple repeating sites known as spectrin-like repeats (Blake, Weir, Newey, & Davies, 2002; Koenig, Monaco, & Kunkel, 1988). There are three independent promoters that have been characterized within this gene in mouse namely brain (B), muscle (M), and Purkinje (P) promoter (Makover, Zuk, Breakstone, Yaffe, & Nudel, 1991). These promoters control the gene expression in various tissues and upon activation, it splices the gene into 78 exons (Chelly et al., 1990).

The dystrophin gene is known to strengthen the muscle and prevent it from injuries especially during the muscle contraction whereas its distinct role in the nerve cells is yet to be elucidated (Garcia-Pelagio, Bloch, Ortega, & Gonzalez-Serratos, 2011). Moreover, DMD which serves as a membrane cytoskeletal protein (Hoffman & Kunkel, 1989); exists abundantly in the heart and the skeletal muscle and relatively low in the brain (Monaco et al., 1986). Mutation in the dystrophin gene may lead to muscular diseases and cardiomyopathy which is found exclusively in males (Hoffman & Kunkel, 1989). The commonly reported dystrophin associated disease is the Duchene muscular disease (DMD) and the Becker muscular disease (BMD), which differ in the percentage of deletion in the gene (Darras, Miller, & Urion, 1993). It is an X-linked disease that is most commonly seen in male, with a ratio of one in 3600-6000 male neonates (Bushby et al., 2010).

The dystrophin interacts with the glycoprotein in the sarcolemma to form a dystro-glycoprotein complex (DGC) which includes dystroglycan, sarcoglycan and utrophin with its possible functional role as listed in the Table 2.3 (Matsumura and Campbell, 1994; Tinsley et al., 1994). Straub and Campbell (1997) have reported the importance of DGC as the disruption of DGC may lead to muscular dystrophies and could also cause damage in the muscle cell feasibility. In addition, the disruption in the DGC also contributes to cancer induced wasting (Acharyya et al., 2005).

Dystroglycan, the interest of the current study is an essential component in the DGC and is composed of two subunits α and β (Sciandra et al., 2003). It forms a linkage or a bridge between the extracellular matrix and the sub-sarcolemmal cytoskeleton (Campbell, 1995). Moreover, its importance, specifically during early embryonic development is clearly defined as the gene knockout of dystroglycan in mice fails to support the development of the Reichert's membrane completely. (Williamson et al., 1997).

Table 2.3. List of Dystrophin – Glycoprotein Complex identified from rabbit skeletal muscle (*Adapted from Campbell, 1995; reviewed by Matsumura and Campbell, 1994; Tinsley et al., 1994)

Location	Protein	Other Names(s)	Size(kDa)	Gene Location	Function
Extraceullular Matrix	Laminin a2 chain	Merosin	400	6p22-23	Basal lamina component that binds α-dystroglycan
Sarcolemma					
Extracellular	α-Dystroglycan	156DAG	156	3p21	Binds laminin-2 and is involved in linkage of dystrophin to laminin-2
Transmembrane	β-Dystroglycan	43 DAG, A3a	43	3p21	Binds dystrophin and is involved in linkage to laminin-2
	Adhalin	50 DAG, A2, SL50	50	17q21	Unknown
	43 DAG	A3b	43	Unknown	Unknown } May form a subcomplex
	35 DAG	A4	35	Unknown	Unknown }
	25 DAP	A5	25	Unknown	Unknown }
Intracellular	α-Syntrophin	59 DAP1, Syn 1	58	20q11	Binds dystrophin/utrophin
	β1-Syntrophin	59 DAP2, Syn 2	59	16	Binds dystrophin/utrophin
	β2-Syntrophin	59 DAP3, Syn 3	60	8q23-24	Binds dystrophin/utrophin
Cytoskeleton	Dystrophin		427	Xp21	Membrane cytroskeletal protein linking transmembrance glycoprotein complex to F-actin

2.4.5.4 Farnesoic acid O-methyltransferase (FAMeT)

The crustacean mandibular organ has been identified in the early sixties and has been reported to play a vital role in reproduction (Le Roux 1968). The mandibular organ located at the base of the tendon is associated with the posterior abductor muscle of the mandibles (Borst, Wainwright, & Rees, 2002). It is known to secrete proteins including methyl farnesoate (MF) and farnesoic acid (FA) (Laufer, 1987; Tobe, Young, & Khoo, 1989). Farnesoic acid O-methyltransferase (FAMeT) is an enzyme that involves in the final stage of the production of methyl farnesoate from farnesoic acid in crustaceans (Gunawardene et al., 2002; Wainwright, Webster, & Rees, 1998). The early discovery of MF was reported in shrimp, *Metapenaeus ensis* (Gunawardene et al., 2002) and has initiated further research to characterize it in other shrimps and elucidates its possible functional roles. Until present, FAMeT has been characterized in the crustaceans which include *H. americanus* (Holford et al., 2004), *L. vannamei* (Hui, Tobe, & Chan, 2008), *Cancer pagurus* (Ruddell et al., 2003) and *Penaeus chinensis* (Li, Xu, Wang, & Wang, 2013).

The Methyl farnesoate shares the same homologous characteristics with insect juvenile hormone, thus it is proposed to have similar functional role in the reproduction besides growth (Hui et al., 2008). In addition FAMeT is also postulated to influence the reproduction and growth of crustaceans as it is expressed along the molt cycle specifically in the eyestalk and the ventral nerve cord (Gunawardene et al., 2002). The importance of FAMeT in the growth of crustaceans is well defined as the disrupted FAMeT gene being unable to complete the molting process as well as alter other molt regulating hormones including cathepsin and hemocyanin in *L.vannamei* (Hui et al., 2008).

2.3.6.5 Arginine Kinase

Phosphagen kinases which belong to the ATP-guanidino phosphotransferases family are catalytical enzymes that are responsible for the conversion of guanidine phosphagens to an adenosine triphosphate (ATP) through a dephosphorylation process. There are seven types of phosphagen kinases that have been reported to date including arginine kinase, creatine kinase, glycocyamine kinase, hypotaurocyamine kinase, lombricine kinase, opheline kinase and taurocyamine kinase (Suzuki, Kawasaki, Furukohri, & Ellington, 1997). Arginine kinase (AK), is the most abundant phosphagen kinase occurring in invertebrates and which even plays an important role in invertebrates (Forbes et al., 2014). The functional role of AK is homologous to the creatine kinase (CK) found in the vertebrate system with a cohesive role in the energy mechanism (Shofer, Willis, & Tjeerdema, 1997).

Most of the AKs are monomers of 40 kDa, while the other phosphagen kinase, creatine kinase, glycocyamine kinase and lombricine kinase are dimeric, or octameric in the case of mitochondrial CK (Abe, Hirai, & Okada, 2007; Fujimoto, Tanaka, & Suzuki, 2005). AK has been extensively studied in crustaceans including kuruma prawn, *Marsupenaeus japonicus* (Abe et al., 2007), Atlantic blue crab, Callinectes sapidus (Holt & Kinsey, 2002), horseshoe crab, *Limulus polyphemus* (Strong & Ellington, 1995), sea Chinese shrimp, *Fenneropenaeus chinensis* (Yao, Wu, Xiang, & Dong, 2005), greasyback shrimp, *Metapenaeus ensis* (Wang, Zheng, Lei, Pan, & Zou, 2009), and *Litopenaeus vannamei* (Yao, Ji, Kong, Wang, & Xiang, 2009). In our previous study, AK of *M. rosenbergii* has also been characterized which shows 96% identity with the AK-1 from cherry shrimp, *Neocaridina denticulate* (Arockiaraj et al., 2011).

The functional role of AK in the crustaceans has been reported with the following functions that are: an energy supplier during hypoxia in *M. japonicas* (Abe et al., 2007) and *C. destructor* (Morris, van Aardt, & Ahern, 2005), protein concentration alteration upon laminarin injection (Yao et al., 2005) and immune response and regulation of protein in *F. chinensis* (Li, Tang, Xing, Sheng, & Zhan, 2014), regulation of energy coupling and immunological response in *L. vannamei* (Yao et al., 2009), alteration of protein expression upon different cadmium levels in *E. sinensis* (Silvestre et al., 2006) and osomoregulation upon salinity changes in *C. sapidus*. In *M. rosenbergii* we have reported AK to have been involved in the production and utilization of energy and elicit immune response upon IHHNV challenge (Arockiaraj et al., 2011).

2.4.5.6 Cyclophilin

Cyclophilin is an 18 KDa cytosolic protein and an immune related gene that belongs to the immunophilin family (Handschumacher, Harding, Rice, Drugge, & Speicher, 1984). The cyclophilin poses an evolutionarily conserved peptidylprolyl cis-trans isomerase (PPIase domain) from yeast to human and poses a diverse functional role in the system (Fischer, Wittmann-Liebold, Lang, Kiefhaber, & Schmid, 1989; Takahashi, Hayano, & Suzuki, 1989). The number of isomers and associated cyclophilin proteins varies for every species. Seven isomers with 16 proteins have been characterized for human whereas nine isomers have been characterized for *Drosophila* (Wang & Heitman, 2005). Cyclophilin A (CypA) is the first member of cyclophilin that has been characterized for mammals and extensively studied in other species (Wang & Heitman, 2005). It is known to regulate the inflammatory process in a disease associated regulation which includes the vascular smooth muscle cell (Jin et al., 2000) and rheumatoid arthritis (Billich, Winkler, Aschauer, Rot, & Peichl, 1997). The CypA is also associated with the protein folding

(Stamnes, Rutherford, & Zuker, 1992), serving as a molecular chaperone (Helekar, Char, Neff, & Patrick, 1994) and as a potential immunosuppressive drug of cyclosporine A (Ke, Zhao, Luo, Weissman, & Friedman, 1993).

CypA in aquatic species is closely associated with innate immune system (Jung et al., 2011b). Studies shows that CypA has been characterized for *P. monodon* (Qiu et al., 2009) and *E. sinensis* (Wang, 2013) and has been testified of its importance in the immune system through mRNA alteration upon pathogen infection. Besides that, the transcriptome analysis of *P. trituberculatus* (Lv et al., 2014) and *M. rosenbergii* (Jung et al., 2011) have helped postulate cyclophilin as a potential candidate for growth and development. Moreover CypA role in growth has also been reported in the Atlantic pink shrimp *Farfantepenaeus paulensis* (Kamimura et al., 2008) and *P.monodon* (Tangprasittipap, 2010) as the mRNA transcripts have been observed to influence body weight.

2.4.5.7 Acyl CoA Desaturase (SCD-1)

Acyl CoA desaturase (SCD-1) is a lipogenic enzyme which belongs to the oxidoreductase family and has been used to produce a monounsaturated fatty acid especially oleic acid from the saturated fatty acid, stearic acid (Ntambi & Miyazaki, 2003). This hydrogen-donor:oxygen oxidoreductase is also commonly known as delta9-desaturase, acyl-CoA desaturase, fatty acid desaturase, and stearoyl-CoA. SCD-1 plays a vital role in the lipogenesis (Figure 2.12), a hydrogen releasing pathway to synthesize fatty acid, which takes place in the cytosol and in the presence of cytochrome b5, NADH (P)-cytochrome b5 reductase, and molecular oxygen (Ntambi, 1999).

SCD-1 is evolutionarily conserved from animal to plant and yeast (Dobrzyn et al., 2004). In general, the SCD-1 has a functional role in the desaturation of saturated fatty acyl –CoA especially palmitoyl- and stearoyl-CoA (Enoch & Strittmatter, 1978). In addition, it also regulates the growth and differentiation as it influences the membrane fluidity and signal transduction (Ntambi, 1999). The first SCD-1 in crustaceans was characterized in *Eriocheir sinensis* from the hepatopancreas and has been reported with an elevated regulation from the megalopa stage to the maturity stage (Guo et al., 2013). Moreover, it has also been reported that the biosynthesis pathway of a long chain polyunsaturated fatty acid varies among crustaceans (Monroig, Tocher, & Navarro, 2013). Besides, the SCD-1 influence on the tumorigenesis is also being the interest of research among vertebrates (Igal, 2010).

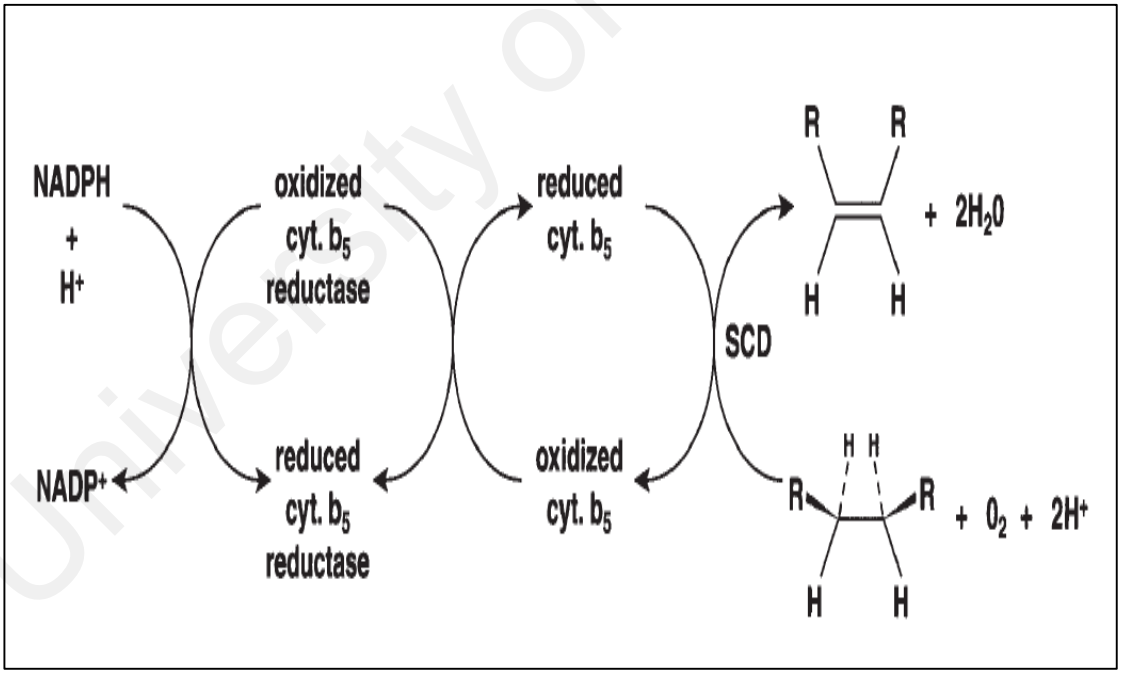


Figure 2.12. Pathway of electron transfer in the desaturation of fatty acids by stearoyl-CoA desaturase*

* Adapted from Ntambi, 1999

CHAPTER 3

MYOSTATIN: IDENTIFICATION AND BIOINFORMATICS CHARACTERIZATION

3.1 INTRODUCTION

Myostatin (MSTN) is also known as a growth differentiation factor-8 (GDF-8) well-known to serve as a negative regulator which inhibits or restricts the growth and development of muscle in vertebrates (Acosta et al., 2005). It belongs to the family of transforming growth factor-β (TGF-β) which exists abundantly in an inactive form in the cytoplasm. MSTN was first identified and has been reported to initiate an increase in the muscle mass as MSTN was disrupted in mice (McPherron et al., 1997). Furthermore, the MSTN role as a negative growth regulator has been supported with the "doubling muscle" mass effect following a mutation in the MSTN observed in a Belgian Blue Cattle Breed (BBCB) and a Piedmontese cattle (Grobet et al., 1997; Kambadur, Sharma, Smith, & Bass, 1997; McPherron & Lee, 1997). In addition, the blocking agents including propeptide and follistatin are also capable of inhibiting myostatin thus promoting muscle growth (Lee & McPherron, 2001). As a whole, the disruption in the function of myostatin either by natural mutation or myostatin gene knockout promotes an increase in the muscle mass by either hyperplasia, hypertrophy or both.

Since the initial discovery, this growth regulating gene with a unique characteristics has begun to be isolated and characterized for other species especially in vertebrates including human, chickens, pigs and fishes (Kocamis, Kirkpatrick-Keller, Richter, & Killefer, 1999; Perez-Montarelo et al., 2014; Schuelke et al., 2004; Stinckens et al., 2005; Wagner et al., 2005; Xu, Wu, Zohar, & Du, 2003). The molecular cloning and characterization of the MSTN gene is essential to provide much needed information

for further application in the aquaculture industry. Following an extensive study of MSTN in vertebrates, it has initiated profound research in invertebrates as well. The objective of this study was to clone and characterize MSTN for *M. rosenbergii* which can be served as template for further molecular application to describe the gene at its functional level. Understanding the high economical value of *M. rosenbergii*, studying this gene thus intend to achieve an ideal prawn with an increased muscle mass in similar to the 'double-muscle' mass effect in near future.

3.2 LITERATURE REVIEW

The molecular cloning and characterization of the MSTN gene is essential as it provides information for further molecular analysis including the gene expression studies, the protein analysis and the gene disruption. Though MSTN has been extensively studied in vertebrates as a negative regulator, it has not been well characterized in invertebrates, thus its role still remains unclear. So far, myostatin-like gene has been cloned and characterized in invertebrates such as fruit fly *Drosophila melanogaster* (Lo & Frasch, 1999), bay scallop *Argopecten irradians* (Kim, Mykles, Goetz, & Roberts, 2004), blackback land crab *Gecarcinus lateralis* (Covi et al., 2008), Chinese mitten crab *Eriocheir sinensis* (Kim et al., 2009), morotage shrimp *Pandalopsis japonica* (Kim et al., 2010) and tiger prawn *Penaeus monodon* (De Santis et al., 2011).

MSTN gene is known to be highly conserved throughout evolution and it is composed of three exons and 2 introns (Patel & Amthor, 2005). The exons includes a signal sequence, an N-terminal propeptide domain and a C-terminal domain which is also actively involved in the gene expression (Joulia-Ekaza & Cabello, 2007). The general molecular weight of MSTN is 25 kDa, which is composed of 376 amino acid precursor proteins (Gonzalez-Cadavid et al., 1998). Upon activation, the gene undergoes proteolytic cleavage at the conserved RXXR, a proteolytic recognition site by the pro-peptide convertase that produces an active mature peptide of MSTN (Kim et al., 2010). The mature peptide contains all the conserved cysteine residues that are involved in di-sulphide bonding, a characteristic feature of myostatin-like and inhibin genes (Kim et al., 2010).

Comprehensive research on the MSTN gene in vertebrates show high conservation especially in the C-terminal where the murine, rat, human, porcine, chicken

and turkey MSTN sequences carry an identical sequence (McPherron & Lee, 1997; Xu et al., 2003). In addition, studies on the myostatin-like gene in invertebrates have revealed nine conserved cysteine residues, seven of which contribute to the intra-molecular and inter-molecular disulfide bond in the native homodimer (Kim et al., 2010; MacLea et al., 2010).

The primers used for the amplification of the full length cDNA were synthesized based on the known unigene sequences. The earlier constructed *M. rosenbergii* transcriptome database (Mohd-Shamsudin et al., 2013) contained 102,230 unigenes. Three partial unigene sequences which are homologous to MSTN protein were identified from the constructed transcriptome dataset using the BLASTx search. Based on the three unigene sequences, specific primers were designed to obtain the full length MSTN sequence by applying the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Rapid Amplification cDNA Ends (RACE) technique.

The RACE, is a powerful technique used to obtain the ends of complementary DNA (cDNA) when only partial sequences of a gene are available, thus this technology was applied to obtain the full length of *Mr*MSTN. In short, an adaptor with a defined sequence was attached to one end of the cDNA and then the region between the adaptor and the known sequences were amplified. The PCR amplified product obtained was cloned into an *E. coli* plasmid and the full length cDNA sequence of *Mr*MSTN was generated. RACE experiments involved a critical aspect of primer designing.

The obtained cDNA sequence was used for various bioinformatics analyses. It was subjected to various physio-chemical factors such as molecular weight, isoelectric point, and the protein sequence was derived from the cDNA sequence itself. Various

important structural and functional regions such as the signal sequence, transmembrane region, MSTN-specific motifs and domains were identified from the derived protein sequence. A signal peptide is a short peptide region usually located at the N terminal region which is used to destine the synthesized proteins towards the secretory pathway (Petersen, Brunak, von Heijne, & Nielsen, 2011). The transmembrane region of a transmembrane protein is usually a single alpha helix regions basically constituting of 20 amino acids (Jones, Taylor, & Thornton, 1994). In general, motifs are defined as structural characteristics and domains as the functional characteristics of a protein which are determined by a specific sequence arrangement which usually remain conserved (Bhattacharyya, Remenyi, Yeh, & Lim, 2006).

The alignments were predicted on the basis of hydrophobicity scales of the sequences. Multiple sequence alignment is used to infer the homology especially among the evolutionarily or structurally related residues in a collection of amino acid sequences which play a key role in the activity of the protein (Wallace, Blackshields, & Higgins, 2005). Phylogenetic tree also termed as dendrogram has been constructed so as to analyze the evolutionarily relationship among a set of organisms and the position of the gene of interest in the evolutionary tree (Tamura et al., 2011). A rooted phylogenetic tree reveals the closest organism and their common ancestor in which a known distant organism is maintained as an out-group. Analysis of the three dimensional (3D) model of the derived protein sequence of *Mr*MSTN divulges the structural importance and distance between various major residues.

3.3 MATERIAL AND METHODS

3.3.1 Molecular Cloning and Sequencing of cDNA

3.3.1.1 Primer designing

In order to generate the full length MSTN sequence, four different partial unigene sequences of MSTN were identified from a *M. rosenbergii* transcriptome database (Mohd-Shamsudin et al., 2013) which had been generated utilizing the Illumina's Solexa sequencing technology. The unigenes were then aligned on the Molecular Evolutionary Genetic Analysis – version 4.0 (MEGA) (Tamura, Dudley, Nei, & Kumar, 2007) using a MSTN-like gene from *P. japonica* (GenBank Accession ID: GU130188) as a reference gene to generate a consensus (Table 3.1). In total, twelve pairs of primers (two sets of primer for each unigene denoted as 1 and 2) were designed using the Primer3Blast program (Ye et al., 2012) to obtain the full length of *Mr*MSTN sequence. The full length of the *Mr*MSTN was obtained by RT-PCR and RACE protocol using the designed primers (Table 3.2).

Table 3.1. Unigenes identified from the transcriptome dataset of *Macrobrachium rosenbergii* listed with positions arranged based on the results of multiple sequence alignment where the sequence from *Pandalopsis japonica* has been used as the reference gene.

No	Unigene	Query position	*P. japonica* (subject position)
1	Unigene16954_All	174-351	43-220
2	Unigene9180_All	1-436	218-656
3	Unigene6326_All	1-302	688-989
4	Unigene74700_All	3-158	835-989

Table 3.2. List of primers designed for sequencing of the full length *Mr*MSTN gene. List of primers includes (a) Primers used to perform RT-PCR (Duplicate set denoted as 1 and 2 for each set of primer) (b) Primers used to perform RACE PCR. Corresponding melting temperatures of each primer are also listed parallel to the primer sequence.

(a) RT-PCR	**Sequence**	**$T_A(^0)$**
MSTN A1 F	TTAACTCCACCCGATATGACAGGGG	57.0
MSTN A1 R	GCCGGTTCATCGTCATTATATGGGG	
MSTN A2 F	ACCCGATATGACAGGGGTTGTGATA	57.0
MSTN A2 R	GGCCGGTTCATCGTCATTATATGGG	
MSTN B1 F	CGCCAGCGGGGCTCAGAATT	49.8
MSTN B1 R	GGTTGCGCCCTCGACGACTT	
MSTN B2 F	GCCAGCGGGGCTCAGAATTC	49.8
MSTN B2 R	TCACTGCCACTTGACGGCCCT	
MSTN C1a F	TGCTGTCGTTACCCTCTCTCGG	49.6
MSTN C1a R	CAGGGACCGTGTTTGGCGCT	
MSTN C2a F	GCTGTCGTTACCCTCTCTCGGT	50.0
MSTN C2a R	ACAGGGACCGTGTTTGGCGC	
MSTN C3b F	ATGAACAGCAGCAGCGCCAAA	52.2
MSTN C3b R	GAGCACCCACAACGATCCACG	
MSTN C4b F	AGATGAACAGCAGCAGCGCCAAA	49.6
MSTN C4b R	AGAGCACCCACAACGATCCACGA	
MSTN C5 F	TAGTAGCACCCAAGGTCTACGA	55.3
MSTN C5 R	TAAGAGCACCCACAACGATCCA	
MSTN D1 F	CACACGGAGGAAAGTCGTCGAG	60.6
MSTN D1 R	ACCGAGAGAGGGTAACGACAGC	
MSTN D2 F	CACACGGAGGAAAGTCGTCGAGG	60.6
MSTN D2 R	ACCGAGAGAGGGTAACGACAGCA	
MSTN D3 F	CACACGGAGGAAAGTCGTCGAGGG	60.6
MSTN D3 R	ACCGAGAGAGGGTAACGACAGCAT	
(b) RACE		
5’ RACE R	GCCGGTTCATCGTCATTATATGGGG	57.0
3’ RACE F	TAGTAGCACCCAAGGTCTACGA	55.3

3.3.1.2 Collection of sample for MrMSTN cloning

M. rosenbergii (15-20 g) were obtained from a local prawn farm at Negeri Sembilan, Malaysia. The prawns were handled with care as per the animal ethical procedure which is described here. Formal approval from the related authorities of the University had been taken into account to conduct the study, thus enabling the prawn farm owner to supply the prawns. The prawns were transported to the laboratory in oxygenated polythene bags. In the laboratory, the prawns were acclimatized over a period of one a week. The prawns were reared in 300 L flat-bottom glass tanks by supplying additional aeration and de-chlorinated freshwater. During acclimatization, the water was changed daily and the best water quality conditions (pH 7.1 and Temperature 28 ± 2 °C) were maintained as suggested by Buikema et al (1982). Moreover, during acclimatization, the prawns were fed twice a day at 0900 h and 1600 h with commercial prawn pellets. Following the acclimatization, the prawns were dissected and the muscle tissue were snap-frozen in liquid nitrogen to avoid any RNA degradation and stored the sample at -80 ^{0}C. This step has to be done accurately as it was utilized for RNA extraction. This work was exempted from official approval from the Institutional Animal Care and Use Committee as the prawns are regarded as edible shellfish. Pooled RNA from muscle tissues of three adult *M. rosenbergii* were used for cDNA cloning of the *Mr*MSTN gene.

3.3.1.3 Total RNA isolation and cDNA synthesis

Total RNA was isolated from *M. rosenbergii* tissues using TRIZOL Reagent method following the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). The total RNA was treated with RNase free DNA set (5 Prime GmbH, Hamburg, Germany) to remove the contaminating DNA. The total RNA concentration was measured spectrophometrically at OD 260/280 and OD 260/230 (NanoVue Plus

Spectrophotometer, GE Healthcare UK Ltd, England). First-strand cDNA was synthesized from total RNA by M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA) followed by oligo-dT (20) VN primer as suggested by the manufacturer.

3.3.1.4 Cloning of MrMSTN cDNA

PCR amplifications were carried out using the designed primers (Table 3.2) with the following conditions: 1.5 µM $MgCl_2$, 5 µM 10X Buffer, 10 µM dNTPs, 10 µM forward and reverse primer, (1.25 U /25 µL) Go Taq Polymerase and 50ng cDNA template in a total reaction volume of 10 µL. The PCR profile was as follows: initial denaturation 94°C for 5 m, 35 cycles of 94 °C for 30 s, annealing temperature (Table 3.2) for 30 s, extension 72 °C for 30 s and 1 cycles of final extension at 72 °C for 7 m.

PCR products were separated by gel electrophoresis using 1.5% (w/v) agarose gel and observed on the UV transilluminator after staining with ethidium bromide. The PCR products with expected sizes were purified using a gel extraction kit (Geneall, Seoul, South Korea), ligated into the TA plasmid vector using the pGEM-T Easy Cloning Kit (Promega, Madison, WI, USA) and transformed into a One Shot Top 10 *Escherichia coli* strain (Invitrogen). Nucleotide sequences of the cloned cDNAs were determined using an automated genetic analyzer (ABI Biosystem, Carlsbad, CA, USA).

3.3.1.5 Random Amplified cDNA Ends (RACE)

A 5’ and 3’ RACE was performed using the RACE primers (Table 3.2) to amplify the 5’ and 3’ regions using a 5’ and 3’ RACE Gene Racer Kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. For RACE, a template of 100 ng cDNA, 8 pmol of gene-specific primer each and other components as described above

were used. The thermal cycler was set as follows: 94 °C for 3 m, 35 cycles of 94 °C for 30 s, 55°C for 30 s, 72 °C for 30 s and final extension was 72 °C for 7 m. All products were cloned and sequenced as described earlier.

3.3.2 Bioinformatics Analysis

3.3.2.1 cDNA Analysis

The full length cDNA sequence of *Mr*MSTN was analyzed using DNAssist (ver. 2.2) (Patterton & Graves, 2000) to obtain the open reading frame (ORF), 5' and 3' untranslated region (UTR), translated amino acid sequence and for calculation of physico-chemical properties including the molecular weight and isoelectric point of the deduced protein.

3.3.2.2 BLAST Analysis

BLASTp search was performed using the online BLAST on National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) (Altschul, Gish, Miller, Myers, & Lipman, 1990). The deduced amino acid sequence of MSTN was given as the query sequence and was searched for homologous sequences in protein database. BLAST CDD search was performed to obtain information about the conserved domains present in the deduced amino acid sequence of *Mr*MSTN.

3.3.2.3 Signal Peptide and Transmembrane Prediction

The N-terminal transmembrane sequence was determined by DAS transmembrane prediction program (http://www.sbc.su.se/~miklos/DAS) (Cserzo, Wallin, Simon, von Heijne, & Elofsson, 1997). The presence and location of signal peptide cleavage sites in the amino acid sequence were predicted using Signal P-4.1 Prediction (http://www.cbs.dtu.dk/services/SignalP/) program (Petersen et al., 2011).

3.3.2.4 Motif Search

Characteristic domains, gene specific motifs and other common motifs present in the deduced amino acid sequence were searched on Prosite, a tool from Expasy bioinformatics research portal (http://prosite.expasy.org/scanprosite/) (de Castro et al., 2006).

3.3.2.5 Three Dimensional (3D) Structure Prediction

The three dimensional structure of the deduced amino acid sequence was predicted by I-Tasser (http://zhanglab.ccmb.med.umich.edu/I-TASSER) (Zhang, 2008). I-TASSER generates full length model of proteins by excising continuous fragments from threading alignments and then reassembling them using replica-exchanged Monte Carlo simulations. The predicted three dimensional structures were viewed using PyMOL 0.99.

3.3.2.6 Multiple Sequence Alignment

Multiple sequence alignment was performed using Clustal W version 2.0 program (Larkin et al., 2007) to find out the evolutionarily conserved residues among the different organisms. The sequences were aligned using BLOSUM method with a GAP extension value of 0.5 and GAP Open value of 5 with a GAP distance value of 5. The graphical output of the aligned sequences was created in BioEdit Version 7.1.3.0.

3.3.2.7 Phylogenetic Analysis

The evolutionary history of MSTN was determined using the Neighbor-Joining method in Phylip 3.69 program (Felsenstein, 1989). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Poisson correction

method and are defined as the units of the number of amino acid substitutions per site. Twenty three amino acid sequences were taken for the analysis. All positions containing gaps and missing data were eliminated.

3.4 RESULTS

3.4.1 Identification and Isolation of MSTN in *Macrobrachium rosenbergii*

The present study describes an initial attempt to isolate MSTN from *M. rosenbergii*. This was achieved using gene specific primers (Table 3.1) designed using the consensus generated from aligning all the unigenes which belongs to MSTN that was obtained from the transcriptome data of *M. rosenbergii* (Mohd-Shamsudin et al., 2013). The primers used for the RACE assay have been optimized to the respective annealing temperature (Table 3.2), preceded by cloning and sequencing, thus obtaining the 5' and 3' sequence of MSTN by RACE PCR. The obtained sequence was then subject to bioinformatics characterization.

3.4.2 Bioinformatic Characterization of *Mr*MSTN

The putative *Mr*MSTN sequence was submitted to the NCBI database under the accession number AFP74567. The sequence contains 1619 base pairs (bp) along with an open reading frame (ORF) of 945 bp which encodes 315 amino acids (Figure 3.1). Moreover, the isolated *Mr*MSTN consists of 354 bp 5' untranslated region (UTR) and 320 bp 3' UTR. The domain analysis indicated that the *Mr*MSTN contains a transforming growth factor-β (TGF-β) propeptide domain at the N-terminal between 137 and 189 amino acids (aa) and a mature transforming growth factor beta like domain at the C-terminal between 216 and 311 aa. The *Mr*MSTN sequence contained all the conserved properties at the active mature peptide region including nine cysteine residues (Cys^{207}, Cys^{216}, Cys^{217}, Cys^{244}, Cys^{248}, Cys^{275}, Cys^{276}, Cys^{308} and Cys^{310}) and a proteolytic cleavage region RNRR (Arg^{197}- Asp^{198}-Arg^{199}-Arg^{200}) which match the RXXR consensus site (Figure 3.1).

The predicted amino acid sequence possesses a probable molecular weight of 35.6 kDa with a theoretical pI of 6.82. The protein constituted with 38 negatively charged residues and 37 positively charged residues. The amino acid sequence of putative *Mr*MSTN was used as a query to perform a BLASTp search. The putative *Mr*MSTN exhibited the highest similarity (72%) with *P. japonica* followed by *Litopenaeus vannamei* and *Penaeus monodon* (68%). *Mr*MSTN also showed a high level of identity with other crustacean members such as *Homarus americanus* (64%), *Gecarcinus lateralis* (59%) and *Eriocheir sinensis* (55%). The similarity percentages mentioned above were based on the alignment of 98% query coverage with all the crustaceans sequences mentioned above.

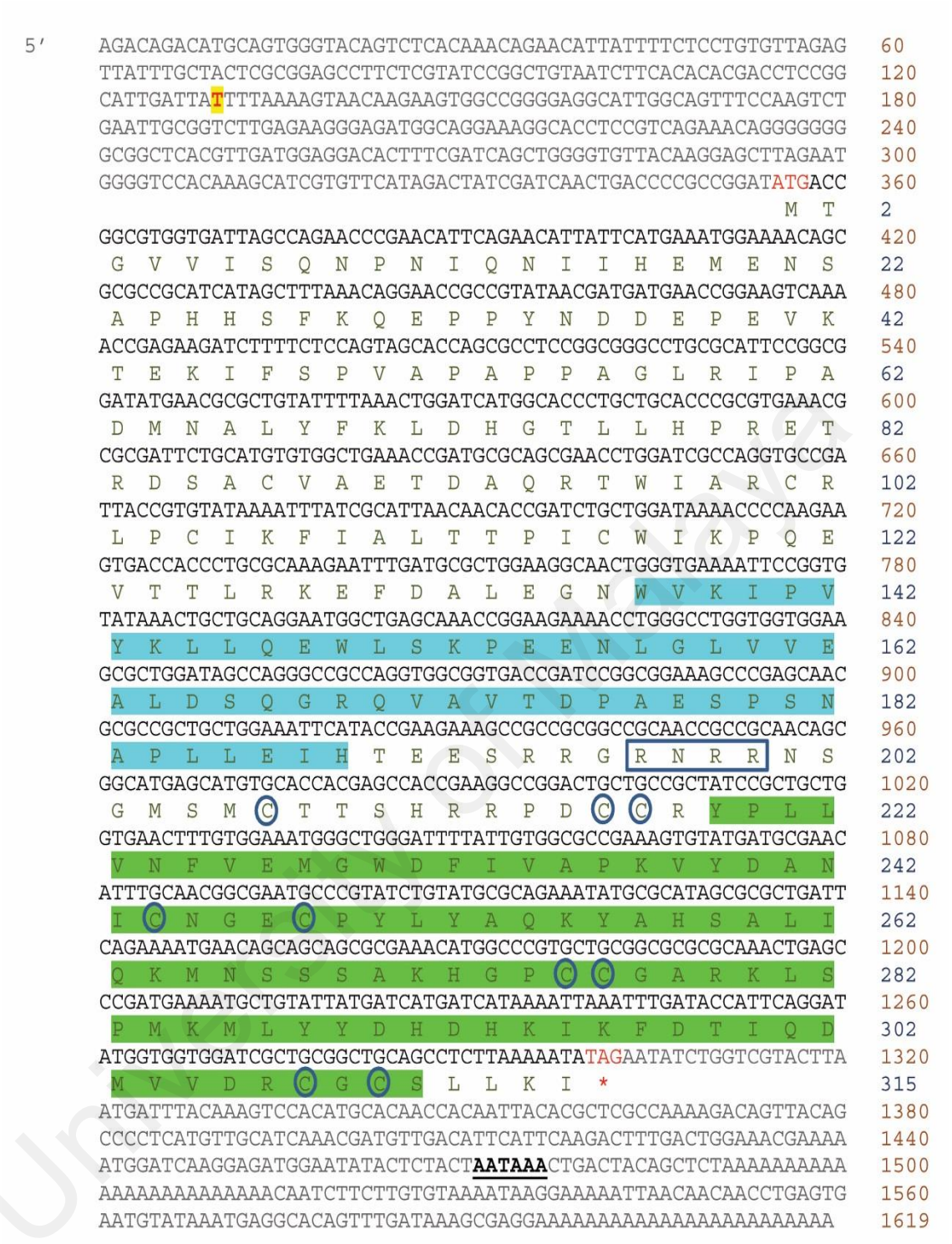
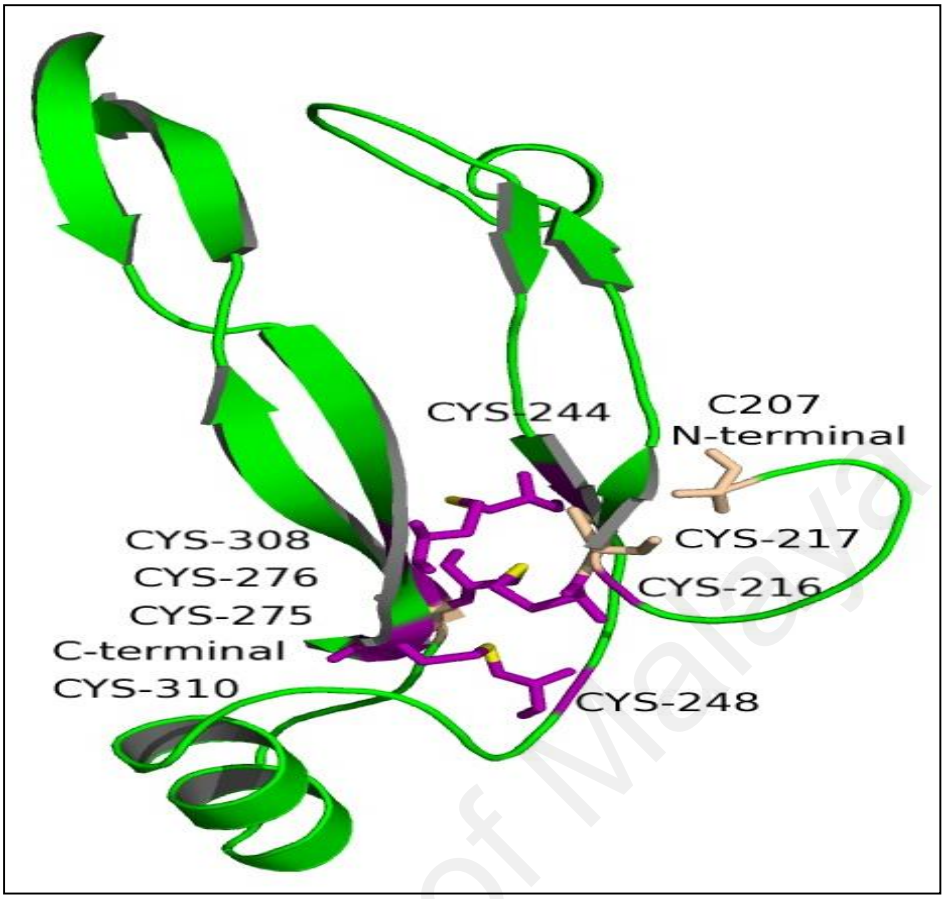


Figure 3.1. Figure represent the nucleotide and the deduced amino acid sequences of *Mr*MSTN (AFP74567). The brown colour numbers indicated on right side of each row represents the position of the nucleotide and the black colour numbers represents the amino acid position. The furin recognition and cleavage site (RXXR) is indicated within the blue box. The conserved seven cysteine residues are indicated in the circle and two additional Cys residues at 207 and 217 also indicated in the circle. The TGF-β propeptide region is highlighted in blue and the TGF- β domain region is highlighted in green. The start and stop codon is highlighted in red. The polyadenylation site is underlined.

The putative *Mr*MSTN also showed similarities with lower and higher groups of vertebrates (42-48%) covering 60% of the query sequence. This includes the lower vertebrates *Tribolium castaneum, Danaus plexippus* and *Salmo salar*; and the higher vertebrates *Sus sucrofa*, *Capra hircus*, *Bos grunniens* and *Homo sapiens*. Moreover, the putative *Mr*MSTN also showed significant levels of similarity to other TGF-β family members, such as the GDF-11 (28%) from *Danio rerio* and *Sparus aurata*. Signal peptide prediction revealed that there is no signal sequence in the deduced amino acid sequence. The transmembrane prediction tool anticipated no transmembrane region thus confirming *Mr*MSTN is not a transmembrane protein.

The 3D structure of protein for putative *Mr*MSTN was constructed using the I-TASSER. The protein model was built for a mature protein domain position at the 207 – 311 amino acids (Figure 3.2A). The position of cysteine (Cys) residues was conserved in all TGF-β families ($Cys^{207,}$ Cys^{216}, Cys^{217}, Cys^{244}, Cys^{248}, Cys^{275}, Cys^{276}, Cys^{308} and Cys^{310}). However, only seven out of nine cysteine residues were involved in the intra and inter-disulphide (S-S) bonding. Three intra-chain S-S bridges were observed at CYS^{216}& CYS^{276}, CYS^{244}& CYS^{308} and CYS^{248}& CYS^{310} (Figure 3.2B). On the other hand, CYS^{275} was shown to be involved in inter-molecular di-sulfide bonding, where it forms the heterodimer of MSTN. Additional cysteine residues (Cys^{207} and Cys^{217}) present in *Mr*MSTN were similar to vertebrate inhibin and myostatin-like factors but did not show any participation in the disulphide formation.

A



B

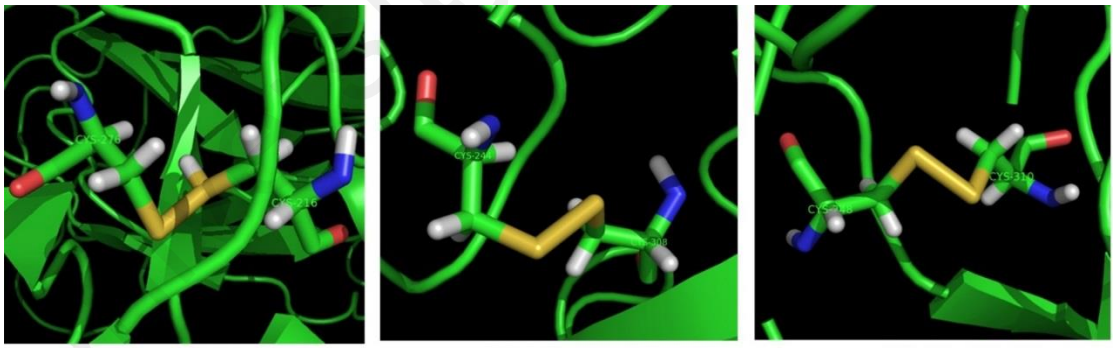


Figure 3.2. Figure represents the three dimensional (3-D) modelling of MSTN for *M. rosenbergii*. **A**. 3-D structure of protein for putative MSTN build for amino acids at positions, 207 to 311 using a crystal structure of the myostatin: follistatin 288 complex as a base template in I-TASSER. All the cysteine residues found within the mature peptide are labelled as 'CYS' with their respective amino acid positions. Inter and intra disulphide bonding is shown in purple colour while intra disulphide bonding connections are highlighted in yellow. **B**. Close-up view of disulphide bonds formation between residues C^{244} and C^{308}; C^{248} and $C^{310}$and C^{216} and C^{276}.

Multiple amino acid sequence alignment of the putative *Mr*MSTN was performed against various crustacean MSTN family members including the *Gecarcinus lateralis, Eriocheir sinensis, Pandalopsis japonica, Litopenaeus vannamei, Penaeus monodon* and *Homarus americanus*; as well as against MSTN from the vertebrate family groups that include *Capra hircus, Homo sapiens* and GDF-11 from *Danio rerio* (Figure 3.3). The alignment shows high conservation at the C-terminal region with many structural characteristics. These include the proteolytic cleavage site, RXXR and nine cysteine residues which are perfectly conserved among all the aligned sequences. A loop region contributing to the structural divergence was identified at the position 271 aa in the putative *Mr*MSTN, and showed alignment with other MSTNs and GDF-11 from different species. The multiple sequence alignments also showed that the last nine residues in the mature peptide, 'MVVDRCGCS' (Met-Val-Val-Asp-Arg-Cys-Gly-Cys-Ser) were conserved in the alignment.

The phylogenetic tree was built to gain insight into the evolutionary relationship of the deduced *Mr*MSTN with other MSTNs, thus a range of animals from invertebrates to higher vertebrates of TGF-β superfamily (GDF-11) were selected. In general, the tree shows that all the crustacean MSTNs were clustered within a single clade with good bootstrap support of 100%. Furthermore, the tree illustrates that *Mr*MSTN possesses a very close phylogenetic relationship of 85% bootstrap value with MSTN from *L. vannamei* and *P. monodon* (Figure 3.4). The current established *Mr*MSTN further clusters with a cast of crabs which then form a sister group with a lobster, *H. americanus*. Overall, 85% frequency was observed between *Mr*MSTN and the MSTN of other species which fall into four major clusters namely higher vertebrates, members of other TGF-β families (GDF-11), lower vertebrates and other arthropods.

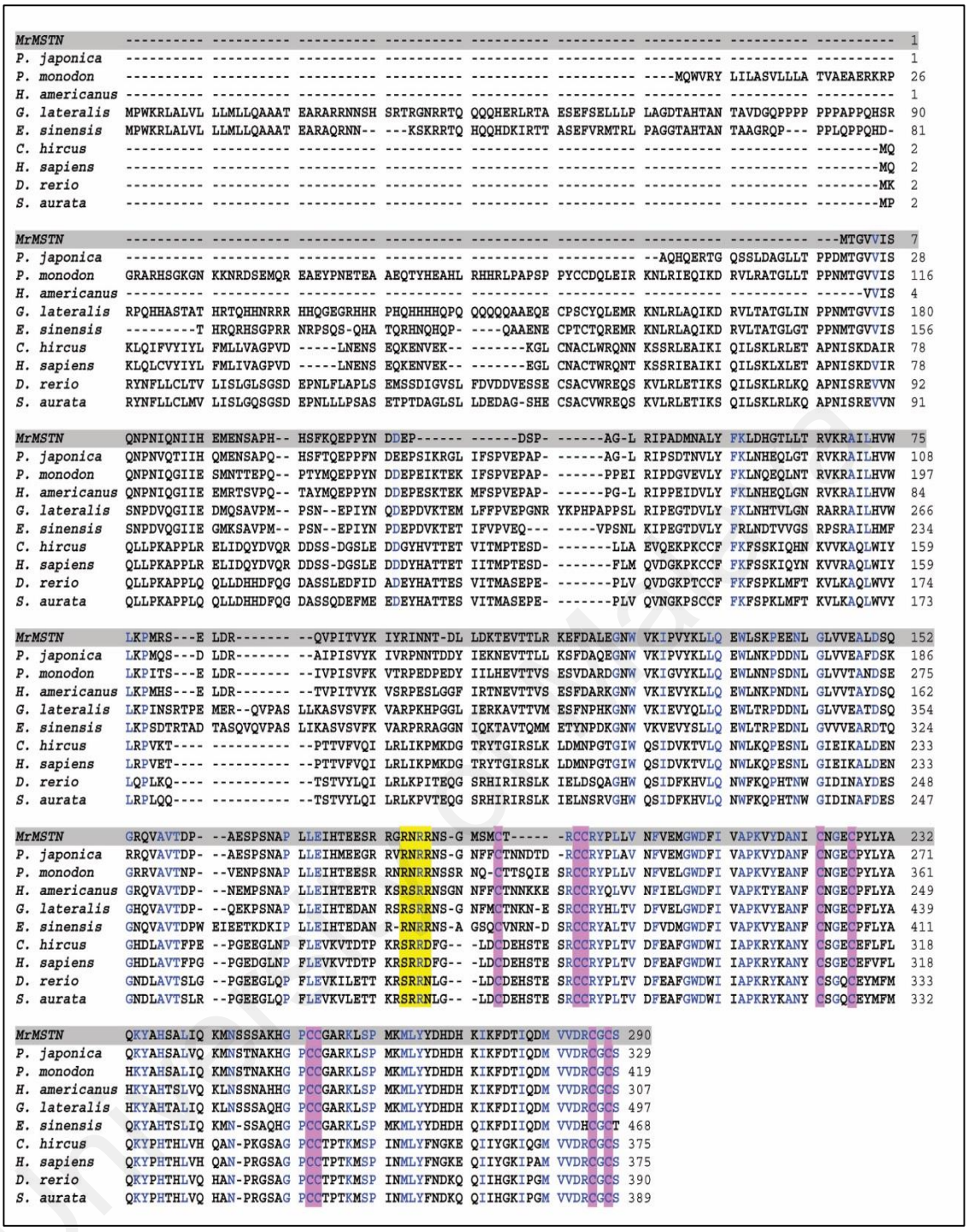


Figure 3.3. Figure representing multiple sequence alignment of *Mr*MSTN amino acid sequences with other homologous of MSTN from *Pandalopsis japonica*, *Penaeus monodon Homarus americanus*, *Gecarcinus lateralis*, *Eriocheir sinensis*, *Capra hircus* and *Homo sapiens* and growth differentiation factor 11 (GDF11) from *Danio rerio* and *Sparus aurata*. In the Figure, the query sequence is highlighted in grey colour. Conserved residues are indicated as blue letters. Moreover, conserved RXXR region is highlighted in yellow and conserved cysteine residues are highlighted in pink. GenBank accession IDs of the sequences used for the alignment is followed: *P. japonica* (ADK62522), *P. monodon* (ADO34177), *H. americanus* (ADK79107), *G. lateralis* (ACB98643), *E. sinensis* (ACF40953), *C. hircus* (AFX59924), *H. sapiens* (ABI48426), *D. rerio* (NP_998140) and *S. aurata* (ACY78404).

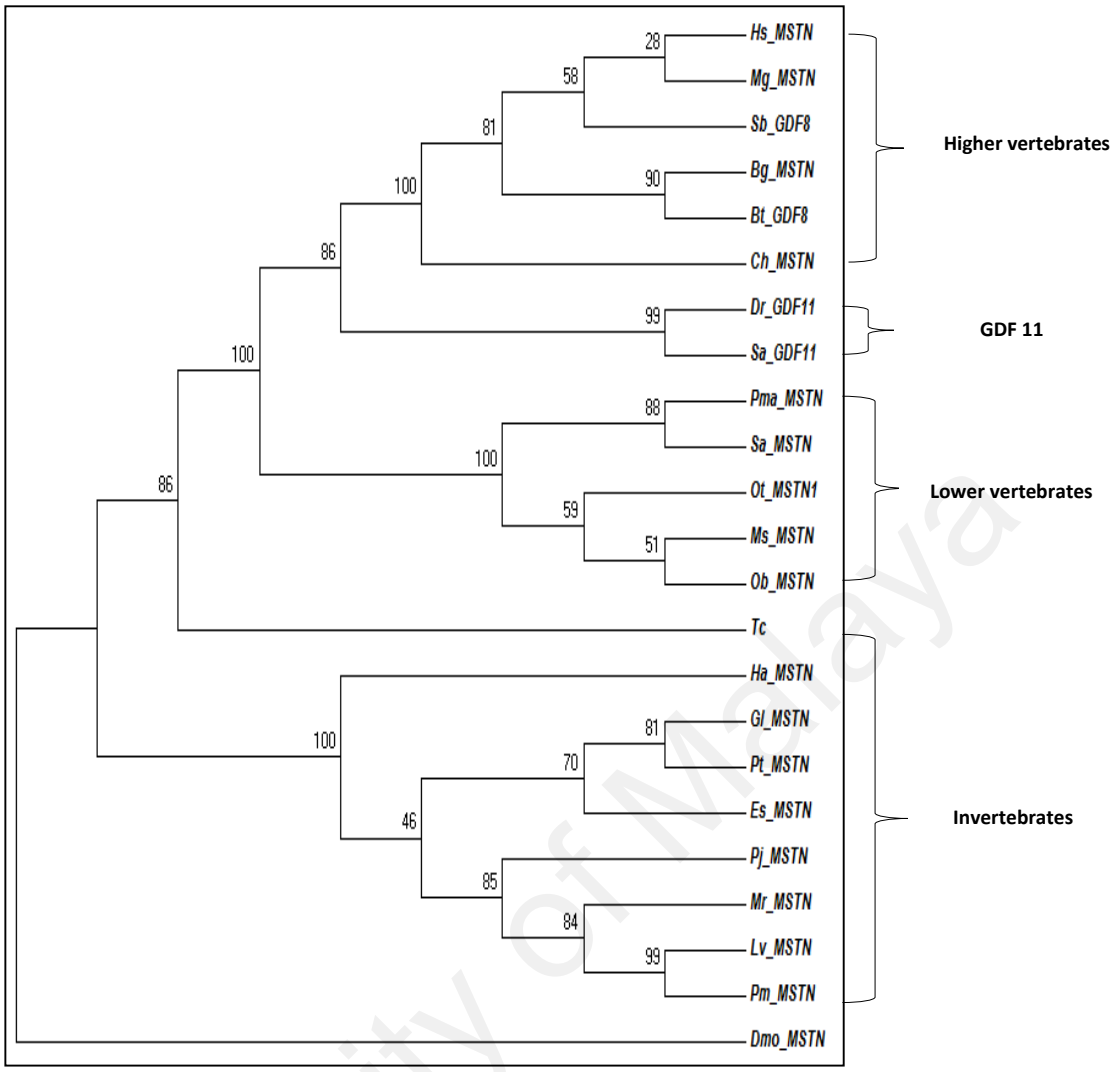


Figure 3.4. Phylogenetic analysis of TGF-β family members, constructed using Neighbour-Joining method on Phylip (ver. 3.69) program. The deduced amino acid sequences were used to construct the tree and bootstrap values are indicated at each node. GenBank accession IDs of the sequences used for the alignment is followed: *Hs*_MSTN, *Homo sapiens* MSTN (ABI48426); *Mg*_MSTN, *Meleagris gallopavo* MSTN (AAB86692); *Sb*_GDF8, *Saimiri boliviensis boliviensis* GDF8 (XP_003940871); *Bg*_MSTN, *Bos grunniens* MSTN (ABY74330); *Bt*_GDF 8, *Bos taurus* GDF 8 (NP_001001525); *Ch*_MSTN, *Capra hircus* MSTN (ADD12958); *Dr*_GDF11, *Danio rerio* GDF11 (NP_998140); *Sa*_GDF11, *Sparus aurata* GDF 11 (ACY78404); *Pm*_MSTN, *Pagrus major* MSTN (AAX82170); *Sa*_MSTN, *Sparus aurata* MSTN (AAK53544); *Ot*_MSTN, *Oncorhynchus tshawytscha* MSTN (ABS44976); *Ob*_MSTN, *Odontesthes bonariensis* MSTN (ADI44159); *Ms*_MSTN, *Morone saxatilis* MSTN (AAK67983); *Ob*_MSTN, *Odontesthes bonariensis* MSTN (AEQ77275); *Tc, Tribolium castaneum* (XP_966819); *Ha*_MSTN, *Homarus americanus* MSTN (ADK79107); *Gl*_MSTN, *Gecarcinus lateralis* MSTN (ACB98643); *Pt*_MSTN, *Portunus trituberculatus* MSTN (ADV78228); *Es*_MSTN, *Eriocheir sinensis* MSTN (ACF40953); *Pj*_MSTN, *Pandalopsis japonica* MSTN (ADK62522); *Lv*_MSTN, *Litopenaeus vannamei* MSTN (AEY11334.1); *Pm*_MSTN, *Penaeus monodon* MSTN (ADO34177); *Dm*_MSTN, *Drosophila mojavensis* MSTN (XP002011400).

3.5 DISCUSSION

MSTN is a well-known growth regulating gene which serves as a negative regulator. It restricts muscle proliferation and growth development in vertebrate systems (Patel & Amthor, 2005). The first MSTN from invertebrates was reported in *Drosophila melanogaster* (Lo & Frasch, 1999) followed by *Argopectan irradians* (Kim et al., 2004). Hence, this gene has gained interest in crustacean research; however, its ultimate role in crustaceans is yet to be clearly defined, hence in-depth research is required to elucidate its complete functionality.

Recently, MSTN has been characterized in invertebrates including *L. vannamei* (Qian et al., 2014), *P. monodon* (De Santis et al., 2011), *P. japonica* (Kim et al., 2010), *E. sinensis* (Kim et al., 2009) and *G. lateralis* (Covi et al., 2008). Overall, the mature peptide of MSTN in invertebrates present within the range of approximately 110 aa and it's domain cysteine residues and proteolytic cleavage site RXXR have been observed to be mostly conserved.

This study reports the first successful cloning and characterization of the MSTN gene from *M. rosenbergii*. Gene characterization is useful to proceed further with molecular analysis that includes gene profiling and gene manipulation. The isolated *Mr*MSTN gene consists of 1619 base pairs (bp), with an ORF of 945 bp that encodes 315 amino acids. The TGF-β family exhibits common conserved properties such as the proteolytic cleavage site, RXXR and cysteine residues which have also been retained in the *Mr*MSTN. Overall, the characterization studies clearly indicate that the currently isolated *Mr*MSTN belongs to the crustacean MSTN group and it is clustered well among the MSTN and GDF 11 of vertebrates.

We have reported that *Mr*MSTN has shown a good alignment with other MSTN including crustaceans and vertebrates and other TGF-β family members which are in accordance with many earlier findings (Covi et al., 2008; De Santis et al., 2011; Kim et al., 2004; Kim et al., 2009; Lo & Frasch, 1999; Qian et al., 2013). *Mr*MSTN is characterized to be conserved structurally with other MSTNs. The conserved characteristics features of the functional properties in this species are similar to the functional role of MSTN in vertebrates (Acosta et al., 2005; Kerr, Roalson, & Rodgers, 2005; Kocamis et al., 1999; Perez-Montarelo et al., 2014; Stinckens et al., 2005; Xu et al., 2003). Moreover, the high conservation observed also supports the previous hypothesis suggesting that the crustacean MSTN is primitive compared to the vertebrate MSTN (Kim et al., 2010).

Despite its conserved nature, the crustacean species have been seen to cluster in a different sub-clade that is well distinguished from the other groups of species. This suggests the potentiality of the gene to be developed as a growth marker. Recently, MSTN has been postulated to be a potential candidate gene related to growth thus suitable to be developed as an SNP marker (Jung et al., 2011). Moreover, potentiality of MSTN as an SNP marker has also been reported in vertebrates including *salmo salar* (Penaloza, Hamilton, Guy, Bishop, & Houston, 2013), *Takifugu rubripes* (Wang et al., 2014), *Cyprinus carpio L* (Sun, Yu, & Tong, 2012) and *Chlamys farreri* (Wang, Meng, Song, Qiu, & Liu, 2010).

The similarity of the characterized *Mr*MSTN with GDF 11 suggests that the MSTN might participate in multiple roles. This was supported by the observation of the conserved alignments between MSTN from crustaceans and other species as well as GDF 11 (Covi et al., 2008; De Santis et al., 2011; MacLea et al., 2010). Besides the known

distinct functional role of MSTN and GDF-11 in vertebrates, it has also been reported that the regulation of both genes are influenced by the common binding proteins including Growth and differentiation factor (GDF) -associated serum protein-1 (GASP-1) and GASP-2 (Lee & Lee, 2013). However, further studies to elucidate all the possible functional roles of MSTN specifically in invertebrates is crucial to gain a better understanding of the gene.

3.6 CONCLUSION

In conclusion, the complete sequence of *Mr*MSTN has been successfully identified from *M. rosenbergii*. The *Mr*MSTN carries all the conserved characteristics belonging to TGF-β family such as cysteine residues and 'RXXR' proteolytic cleavage region. The *Mr*MSTN showed high conservation specifically in mature peptide domain, throughout the evolution from vertebrates to invertebrates as well as with GDF-11. This suggest that MSTN could carries multiple functional roles. Thus there is an urge for further molecular approach to elucidate the possible roles of MSTN, especially in invertebrates. The role of *Mr*MSTN, representing the invertebrates was further characterized molecularly which is discussed in the following chapters.

CHAPTER 4

GENE PROFILING OF *Mr*MSTN FOR *Macrobrachium rosenbergii*: TISSUE DISTRIBUTION, mRNA TRANSCRIPTION AT DIFFERENT LIFE STAGES AND UPON IHHNV INFECTION

4.1. INTRODUCTION

The investigation of the expression of gene families that encode proteins which control growth and development of various organisms have enabled the understanding of their functional roles. The mRNA regulation studies following the gene identification and characterization has been standardized as an initial protocol to predict the role of a gene at the molecular level. Gene expression studies aid to quantify expression level of a particular gene. The gene quantification is an easy, reliable and efficient way of assessing gene expression in which the expression of the gene of interest is compared to the expression of an internal control gene (Bustin et al., 2009). The quantitative real-time PCR (qRT-PCR) technique is applied to relatively quantify the amount of mRNA transcripts present in a particular tissue.

Following the discovery of the MSTN gene, gene expression study has been conducted to record the mRNA regulation patterns during various developmental stages of the tissues and the stem cells especially in animals as well as in humans (McKay, Ogborn, Bellamy, Tarnopolsky, & Parise, 2012; Roberts & Goetz, 2001; Schuelke et al., 2004; Shibata et al., 2006; Tsao et al., 2013). The MSTN regulation pattern shows the preliminary role of the gene serving as a negative growth regulator in vertebrates. Although MSTN has been extensively studied in vertebrates as a negative regulator, the information is limited in invertebrates. The mRNA profiling has been also conducted in invertebrates specifically crustaceans during the different molting stages (Covi, Bader, Chang, & Mykles, 2010; Kim et al., 2010; MacLea et al., 2010) as well as at the different

larval stages (Qian et al., 2013). The earlier reports suggested the role of MSTN to be similar as in vertebrates as it restrict the growth development upon presence (Covi et al., 2010; Covi et al., 2008; Kim et al., 2010; MacLea et al., 2010). However, the contradicting role of MSTN as it favors the muscle growth has also been reported in *P. monodon* (De Santis et al., 2011) and *L. vannamei* (Qian et al., 2013) which have put the overall role of MSTN in invertebrates in controversy.

Hence, it is necessary to elucidate the potential role in prawns more precisely. The fundamental information on, knowing about MSTN distribution in tissues, various life stages of *M. rosenbergii* and its regulation upon infection will provide insights into its importance in the prawn developmental stages and during the growth affecting infection. Keeping this in view, this study was carried out to demonstrate the gene expression profile of MSTN from healthy, IHHNV infected and at various developmental stages of *M. rosenbergii*. The present study provides basic information of the MSTN mRNA profiling for *M. rosenbergii* alongside establishing the preliminary report of its influence in growth progression and correlation with a growth affecting infection. This will be very useful in developing the gene as a biomarker and to enhance growth which could influence the turnover of the production in the aquaculture industry.

4.2. LITERATURE REVIEW

Information on the molecular processes that regulate growth and development in human and animal has been greatly expanding in recent years (Perez-Montarelo et al., 2014). The prawns regularly undergo a process called molting during which they cast their exoskeleton in order to grow. Thus, the growth in prawns depends on their efficiency of muscle restoration after each molting process. Therefore research into key regulatory genes that are responsible for muscle restoration is of obvious importance.

MSTN is one of the important genes which is closely related to growth in muscles besides others genes such as crustacean hyperglycaemic hormones (CHH), actin, tropomyosin, ecdysteroids, fatty acid elongase, the crustacean neuropeptide family and the molten inhibiting hormone (MIH) (Jung et al., 2011b). MSTN is identified as a growth differentiation factor-8 (GDF-8) which functions as a negative regulator of growth where it inhibits the growth and development of muscles in vertebrates (Saunders et al., 2006). They belong to the transforming growth factor-β (TGF-β) family which has been also reported to be involved in the growth of shrimp muscles (De Santis et al., 2011; Kim et al., 2009; Kim et al., 2010; Shen, Ren, Zhu, & Zhang, 2015).

Overall, the MSTN gene has been reported to be the control point in enhancing muscle mass during the development of a species (Vianello, Brazzoduro, Dalla Valle, Belvedere, & Colombo, 2003). Previous findings have reported that MSTN was regulated during the growth progression, from the gestation period until aging in vertebrates (Bass, Oldham, Sharma, & Kambadur, 1999). The MSTN regulation at different developmental stages ranging from the embryonic stage to the adult stage has been reported in vertebrates including cattle (Bass et al., 1999; Kambadur et al., 1997; Shibata et al., 2006), porcine (Ji et al., 1998; Stinckens et al., 2005), barramundi (de Santis, Gomes, & Jerry, 2012) and

chicken (Kocamis et al., 1999). In addition, the MSTN has also been regulated during the embryonic and larval development of zebrafish (Vianello et al., 2003).

The existing MSTN mRNA transcripts reported for a range of developmental stages in vertebrates have provoked interest to record similar expression patterns in invertebrates. Studies show that the crustacean's MSTN have been profiled during the differential molting stages (Covi et al., 2010; Kim et al., 2009; Kim et al., 2010; MacLea et al., 2010) as well as during larval development (Qian et al., 2013). The current study emphasizes on profiling the MSTN mRNA transcripts at different tissues and the fundamental life stages in *M. rosenbergii*. The life cycle of *M. rosenbergii* comprises of four stages namely the larval, post-larval, juvenile and adult stages. The growth rate and size of prawns during these four stages varies significantly. Moreover, molting is actively carries out during the transition period from larvae to post-larvae and shows remarkable physical changes following each molting (Nandlal & Pickering, 2005). In addition, Molting is usually associated with a sudden increase in the size and weight of the prawn (Uno, 1969).

Looking specifically into tissues, MSTN mRNA has been reported to be abundant specifically in the muscle tissue (Kim et al., 2010; McPherron et al., 1997). Although the MSTN is highly expressed in skeletal muscle, studies have also affirmed its expression in other tissues including the mammary gland of porcine (Ji et al., 1998), in spleen tissue of mice (Lyons, Haring, & Biga, 2010), in Purkinje fibers and cardiomyocytes of BCBB (Sharma et al., 1999) and in the gonads and mantle of mussel (Nunez-Acuna & Gallardo-Escarate, 2014). Thus, a wide range of studies from vertebrates to invertebrates have reported a ubiquitous MSTN expression in both the

muscle and the non-muscle tissues (Covi et al., 2010; Kim et al., 2010; Nunez-Acuna & Gallardo-Escarate, 2014; Ostbye et al., 2001; Xu et al., 2003).

Realizing the importance of MSTN in growth and development, it has also been further developed to contribute to the therapeutic treatment including muscular disorders and diseases that affect muscle growth and muscle wasting. The common muscular disorder, muscular dystrophy in a mouse model showed an increase in the muscle mass and strength in the absence of the MSTN gene (Patel, Macharia, & Amthor, 2005; Wagner, McPherron, Winik, & Lee, 2002). Moreover, muscle wasting consequence in an AIDS patients has been observed with 10% weight loss, exhibiting higher levels of MSTN compared to a healthy individual (Gonzalez-Cadavid et al., 1998). Thus this project has been also initiated to investigate the regulation of myostatin in *M. rosenbergii* upon challenge with IHHNV infection, in which it's hypothesized to affect the growth of the prawn.

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is a single stranded DNA virus which belongs to the *Parvoviridae* family. It has been reported to cause mortality up to 90% upon inducing infection in prawns such as *Litopenaeus vannamei* and *Litopenaeus* stylirostris, specifically at the juvenile stage (Briggs, 2005; Lightner, 1996). Recently *M. rosenbergii* has been characterized as susceptible to IHHNV viral attack other than which an early detection has also been reported in *L. stylirostris, L. vannamei* and *Peanus monodon* (Hsieh et al., 2006). Additionally, the IHHNV has also been observed in the wild berried *M. rosenbergii* and further transmission to the progeny (Hazreen Nita et al., 2012).

Runt deformity syndrome (RDS) was observed in prawns with a prolonged IHHNV infection and leads to prawn suffocation from growth deficiency (Lightner, 1996). Post-larval and sub-adult stages of prawns is the main target of IHHNV infection in *M. rosenbergii* which leads to consequences such as growth deformities at the 4^{th} to 6^{th} abdominal segments as well as the tail fan (Hsieh et al., 2006). Moreover, IHHNV infection has been reported to induce slow growth in marine shrimps which include *P. monodon* (Rai, Pradeep, Karunasagar, & Karunasagar, 2008), *L. stylirostris* and *L. vannamei* (Bell & Lightner, 1984; Hsieh et al., 2006; Kalagayan et al., 1991; Lightner et al., 1992).

It is therefore vital to gain insights into the relationship between the growth of *M. rosenbergii* and IHHNV infection in an attempt to develop solutions for growth deficiency caused by the infection. MSTN with its known therapeutic potential in vertebrate could be a suitable gene candidate for study in *M. rosenbergii* to be potentially used to overcome the growth deficiency problems. With the knowledge of its regulatory mechanism, MSTN during IHHNV infection could possibly provide fundamental insights to further explore the gene and promote growth despite IHHNV infection in *M. rosenbergii*.

4.3 MATERIALS AND METHODS

4.3.1 Primer Designing

The real time primers were designed using IDT SCI TOOL programs (http://www.idtdna.com/SciTools/SciTools.aspx) (Owczarzy et al., 2008) using a characterized sequence (mature peptide - the functional region) from the previous objective as mentioned in Chapter 3 (Table 4.1).

Table 4.1. List of primers used for gene expression studies along with the sequence and their respective annealing temperature **T_A (^{0})**.

Primers	Sequence	T_A(^{0})
*Mr*MSTN F	TAGTAGCACCCAAGGTCTACGA	60.0
*Mr*MSTN R	TAAGAGCACCCACAACGATCCA	60.0
EF F	ACTGCGCTGTGTTGATTGTAGCTG	60.0
EF R	ACAACAGTACGTGTTCACGGGTCT	60.0

4.3.2 Sample Collection and Processing

4.3.2.1 M.rosenbergii for tissue distribution

M. rosenbergii (15-20 g) were obtained from a local prawn farm at Negeri Sembilan, Malaysia. The prawns were handled with care as per the animal ethical procedure which is described here. Formal approval from the related authorities of the University had been taken into account conduct the study, thus enabling the prawn farm owner to supply the prawns. The prawns were transported to the laboratory in oxygenated polythene bags. In the laboratory, the prawns were acclimatized over a period of one week. The prawns were reared in 300 L flat-bottom glass tanks by supplying additional aeration and de-

chlorinated freshwater. During acclimatization, the water was changed daily and the best water quality conditions (pH 7.1 and Temperature 28 ± 2 °C) were maintained as suggested by Buikema et al (1982). Moreover, during acclimatization, the prawns were fed twice a day at 0900 h and 1600 h with commercial prawn pellets. This work was exempted from official approval from the Institutional Animal Care and Use Committee as the prawns are regarded as edible shellfish. Tissues including hemocytes, eyes, hearts, stomachs, hepatopancreas, gills, intestines, pleopods and muscle were collected from nine adults, immediately snap-frozen in liquid nitrogen and stored at -80 ^{0}C for further pooled RNA extraction. There are 3 sets of pooled RNA sample in total, wherein each set of pooled RNA were obtained from 3 prawns. Then, cDNA were transcribed from the three sets of pooled RNA and further used as template for *Mr*MSTN gene expression profiling study in *M. rosenbergii.*

4.3.2.2 Different life stages in M. rosenbergii

M. rosenbergii at different life stages were collected from a freshwater prawn breeding hatchery at Negeri Sembilan, Malaysia. A pool of larvae (day one) were collected from the hatchery, immediately snap-frozen in liquid nitrogen and then stored at -80 ^{0}C for further RNA extraction. Post-larvae (day 60), juvenile (approximately 5 g) and adult (approximately 15 g) were transported to the laboratory in oxygenated polythene bags. In the laboratory, the prawns were acclimatized over a period of one week. The prawns were reared in 300 L flat-bottom glass tanks by supplying additional aeration and de-chlorinated freshwater. During acclimatization, the water was changed daily and the best water quality conditions (pH 7.1 and Temperature 28 ± 2 °C) were maintained as suggested by Buikema et al (1982). Moreover, during acclimatization, the prawns were fed twice a day at 0900 h and 1600 h with commercial prawn pellets. This work was

exempted from official approval from the Institutional Animal Care and Use Committee as the prawns are regarded as edible shellfish. Muscle tissues from three individuals of each life stage were isolated, immediately snap-frozen in liquid nitrogen and then stored at -80 ^{0}C for further RNA extraction followed by first-strand cDNA synthesis. These three different cDNA templates were then used for further quantitative real-time PCR (qRT-PCR) analysis.

4.3.2.3 M. rosenbergii and IHHNV challenge

M. rosenbergii (15-20 g) were obtained from a local prawn farm at Negeri Sembilan, Malaysia. The prawns were handled with care as per the animal ethical procedure which is described here. Formal approval from the related authorities of the University had been taken into account to conduct the study, thus enabling the prawn farm owner to supply the prawns. The prawns were transported to the laboratory in oxygenated polythene bags. In the laboratory, the prawns were acclimatized over a period of one week. The prawns were reared in 300 L flat-bottom glass tanks by supplying additional aeration and de-chlorinated freshwater. During acclimatization, the water was changed daily and the best water quality conditions (pH 7.1 and Temperature 28 ± 2 °C) were maintained as suggested by Buikema et al (1982). Moreover, during acclimatization, the prawns were fed twice a day at 0900 h and 1600 h with commercial prawn pellets. This work was exempted from official approval from the Institutional Animal Care and Use Committee as the prawns are regarded as edible shellfish.

In order to study the IHHNV induced mRNA expression analysis, the prawns were injected with IHHNV. Briefly, IHHNV infected prawn tail tissue which tested positive by nested PCR was homogenized in sterile 2% NaCl (1:10, w/v) solution and

centrifuged at 3000 rpm for 5 m at 4 ^{0}C. The supernatant was filtered through a 0.45 µm filter and used for injecting (100 µL per 15 g prawn) the animals. Tissues (hemocytes, eyes, hearts, stomachs, hepatopancreas, gills, intestines, pleopods and muscle) from three individuals were collected before (0 h), and post injection (3, 6, 12, 24, and 48 h), immediately snap-frozen in liquid nitrogen and then stored at -80 ^{0}C for further RNA extraction followed by first-strand cDNA synthesis. These three different cDNA templates were then used for further quantitative real-time PCR (qRT-PCR) analysis. Using a sterilized syringe, the hemolymph (0.2-0.5 mL per prawn) was collected from the prawn heart and immediately centrifuged at 3000 rpm for 10 m at 4 ^{0}C to allow hemocyte collection for total RNA extraction. The samples collected were randomly, qualitatively tested for the presence or absence of IHHNV infection by using OIE forward primer (5'-CGGAACACAACCCGACTTTA-3') and reverse primer (5'-GGCCAAGACCAAAATACGAA-3') for a product length of 389 bp fragment (Manual of Diagnosis Tests for Aquatic Animals 2003; 2009).

4.3.3 RNA Purification and cDNA Synthesis

Total RNA was isolated from *M. rosenbergii* tissues using TRIZOL reagent following the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). Total RNA was treated with RNase free DNA set (5 Prime GmbH, Hamburg, Germany) to remove the contaminating DNA. The total RNA concentration was measured spectrophometrically (NanoVue Plus Spectrophotometer, GE Healthcare UK Ltd, England). First-strand cDNA was synthesized from total RNA by M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA) followed by oligo-dT (20) VN primer in accordance with the manufacturer's protocol.

4.3.4 Quantitative Real-Time PCR Analysis of *Mr*MSTN mRNA Expression

Quantitative real-time PCR analysis (qRT-PCR) was carried out using an ABI 7500 Real-time Detection System (Applied Biosystems, Foster City, California, USA) in a 20 µL reaction volume containing 50 ng of cDNA from each tissue, Power SYBR_ Green Master Mix, 0.3 µM of each primer and 7.8 µL dH_2O. The qRT-PCR cycle profile was 1 cycle of 95 ^{0}C for 10 m, followed by 40 cycles of 95 ^{0}C for 10 s and 60 ^{0}C for 1 m. The same qRT-PCR cycle profile was used for the internal control gene and the elongation factor-1 (EF-1). The primers used in this study are presented in Table 4.1. EF-1 primers were designed based on EST similar to elongation factor 1α from *M. rosenbergii* (GenBank Accession No. EL609261). After the PCR program, the data was analysed with the ABI 7500 SDS software (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. The comparative CT method ($2^{-\Delta\Delta Ct}$ method) (Livak & Schmittgen, 2001) was used to analyse the expression levels of *Mr*MSTN. All data was given in terms of relative mRNA expressed by means ± standard deviation.

4.3.5 Statistical Analysis

For comparison of relative *Mr*MSTN mRNA expression, statistical analysis was performed using one-way ANOVA and mean comparisons were performed by Tukey's Multiple Range Test using SPSS 11.5 with a 5% significance level.

4.4 RESULTS

4.4.1 *Mr*MSTN mRNA Expression in Tissues

The presence of *Mr*MSTN mRNA transcript in various tissues of a healthy adult *M. rosenbergii* was quantified by quantitative real-time RT-PCR (Figure 4.1). The result shows *Mr*MSTN was expressed in all the tissues tested. The highest expression was observed in the muscle, followed by the intestine, heart and hemocyte. Meanwhile lower expressions of *Mr*MSTN were detected in the stomach, gills, pleopods and hepatopancreas, whereas eyes showed the lowest expression. A statistical analysis showed that *Mr*MSTN mRNA expression was significantly higher ($P < 0.05$) in the muscle. Therefore, muscle tissue was selected as a target tissue to further quantify the *Mr*MSTN mRNA transcript at different life stages as well as at the post infection stage with IHHNV.

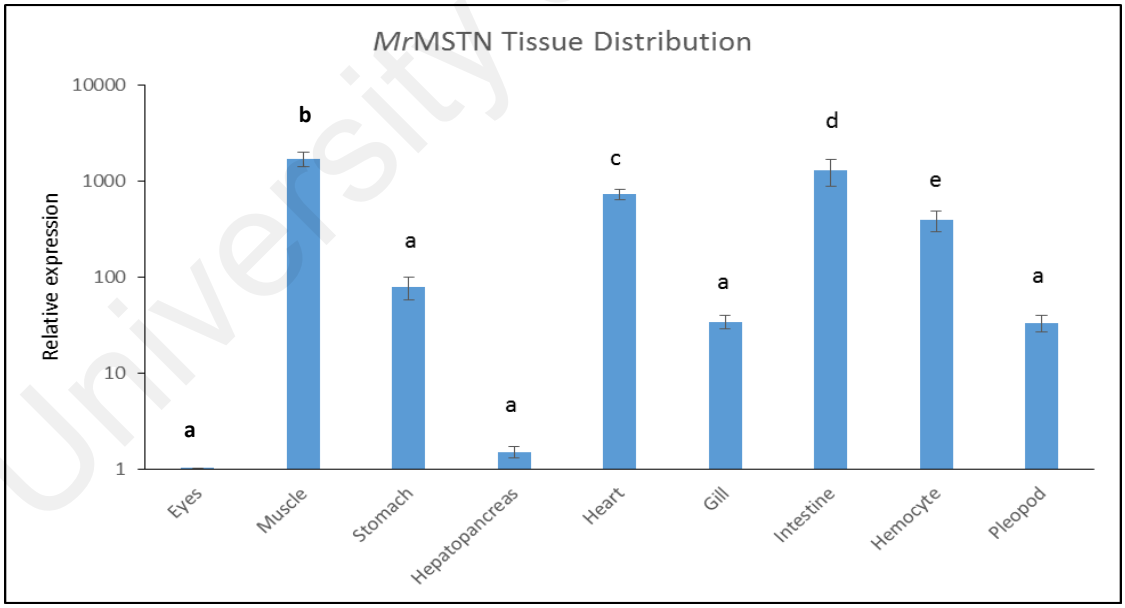


Figure 4.1. Gene expression patterns of *Mr*MSTN in different tissues of *M. rosenbergii* by qRT-PCR. *Mr*_EF (expressing at similar levels in all the tissues) served as constitutively expressed control. The statistical analysis was based on the comparison of the relative expression ratio of the *Mr*MSTN gene to the *Mr*_EF by calculating the $2^{-\Delta\Delta Ct}$ values. Data is expressed as a ratio of *Mr*MSTN mRNA expression in eye. The different alphabets are statistically significant at the $P < 0.05$ by one-way ANOVA and Tukey's Multiple Range Test. Columns denoted with the same letters are not significantly different ($P < 0.05$).

4.4.2 *Mr*MSTN mRNA Expression at Different Life Stages

In this analysis, the expression of *Mr*MSTN at different life stages of *M. rosenbergii* that included larvae, post larvae, juvenile and adult stages (Figure 4.2). The data showed that the expression of *Mr*MSTN significantly ($P < 0.05$) varies at different stages of growth. *Mr*MSTN was observed to be highly expressed during the larval stage, followed by a drastic decrease at the post larval stage. However, expression increased three folds at the juvenile stage followed by a slight declination at the adult stage.

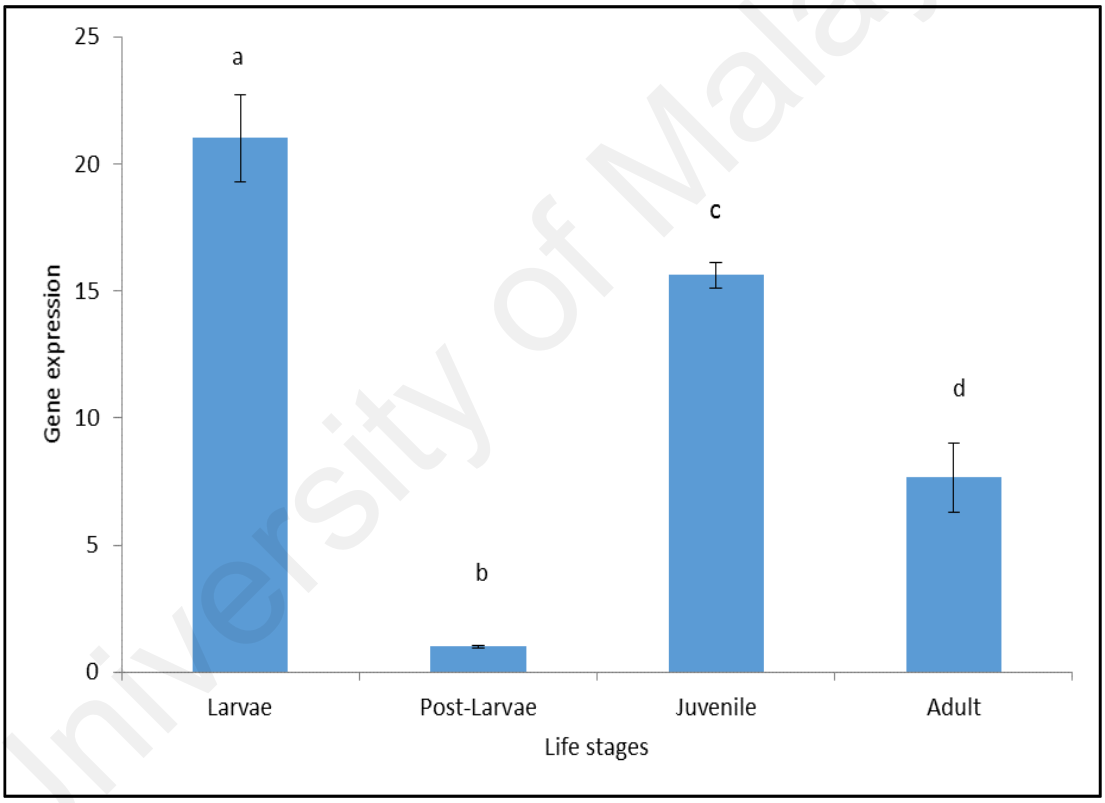


Figure 4.2. Gene expression patterns of *Mr*MSTN in differential life stages of *M. rosenbergii* by qRT-PCR. *Mr*_EF (expressing at similar levels in all tissues) served as constitutively expressed control. The statistical analysis was based on comparison of the relative expression ratio of the *Mr*MSTN gene to the *Mr*_EF by calculating $2^{-\Delta\Delta Ct}$ values. Data are expressed as a ratio of *Mr*MSTN mRNA expression in post – larvae. The statistically significant of *Mr*MSTN expression ($P < 0.05$) between different life stages are indicated as a, b, c and d.

4.4.3 *Mr*MSTN mRNA Expression in Muscle after IHHNV Challenge

Since IHHNV is known to affect growth, this study was conducted to analyse the *Mr*MSTN expression patterns upon IHHNV infection (Figure 4.3). The organisms challenged with IHHNV were randomly tested for IHHNV positivity at different time point using primer set of 389 bp product. The results showed positive signs off IHHNV infection (Appendix B). *Mr*MSTN expression levels upon IHHNV infection showed a gradual increase in the gene expression with a sharp increase at the 6 hour time- point post-injection. The levels of expression have been observed to decrease at 12 hours, followed by a slower decline at 24 hour until 48 hours. Significant differences ($P < 0.05$) in expression were observed at the 3, 6, 12, and 24h time points post-injection between the IHHNV challenged group and the control group.

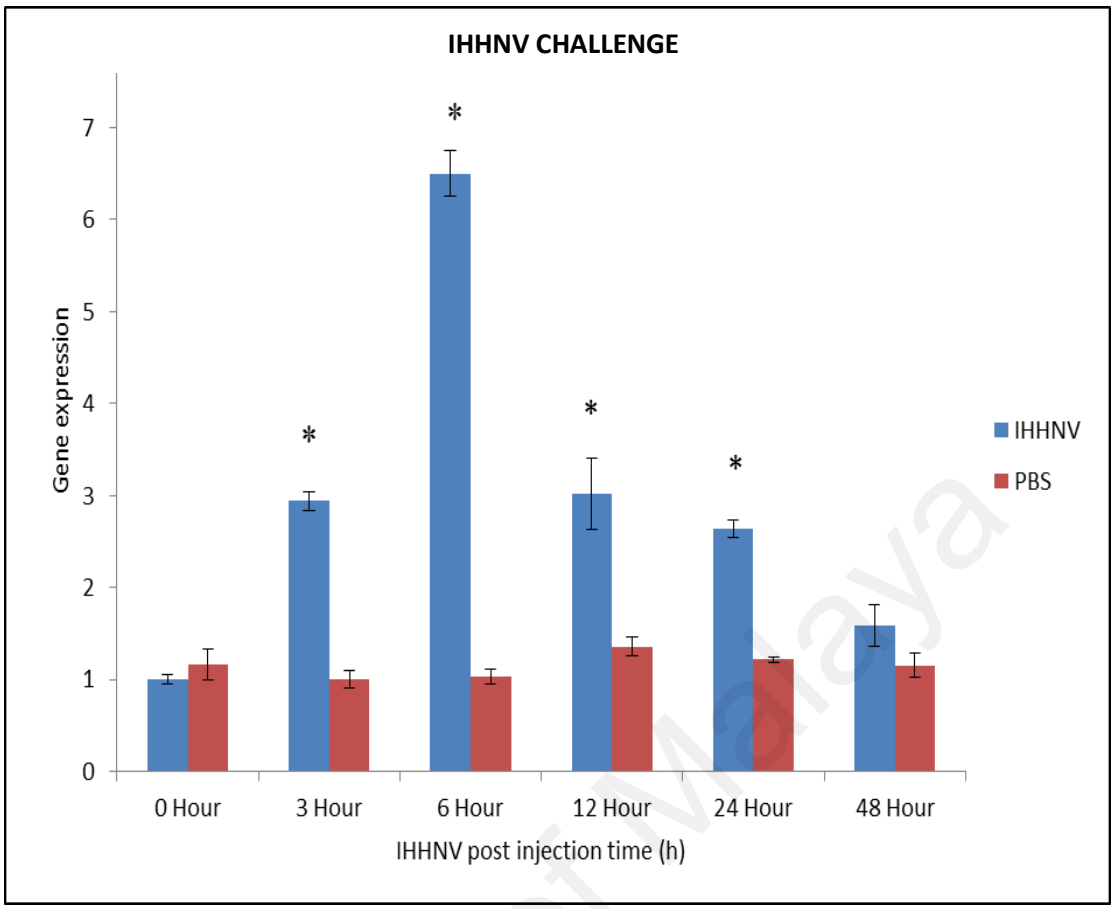


Figure 4.3. Gene expression patterns of *Mr*MSTN injected with IHHNV and PBS (control) in *M. rosenbergii* by qRT-PCR. *Mr*_EF (expressing at similar levels in all tissues) served as constitutively expressed control. The statistical analysis were based on the comparison of the relative expression ratio of *Mr*MSTN gene to the *Mr*_EF by calculating $2^{-\Delta\Delta Ct}$ values. Data are expressed as a ratio of *Mr*MSTN mRNA expression at 0 hour. The statistically significance of *Mr*MSTN expression ($P < 0.05$) between the challenged and the control group are indicated with asterisks.

4.5 DISCUSSION

*Mr*MSTN was expressed in all the tissues tested with the highest mRNA expression in the muscle followed by the heart, intestine and haemocytes. To date, mRNA expression of MSTN in crustaceans has been reported only in *P. japonica, P. monodon, H. americanus, L. vannamei* and *G. lateralis* (De Santis et al., 2011; Kim et al., 2010; MacLea et al., 2010; Qian et al., 2013). The current *Mr*MSTN expression is concurrent with the *Pj*MSTN expression (Kim et al., 2010) in which it reveals higher regulation levels at the muscular tissues including the heart, thoracic muscle, deep abdominal flexor and extensor. Moreover, minimal expression has been reported in non-muscular tissues such as hepatopancreas and gill in *Pj*MSTN (Kim et al., 2010) and *Gl*MSTN (Covi et al., 2008). In contrast, *P. monodon* MSTN expression has been higher in the heart compared to other tissues (Santis *et al*., 2011).

The ubiquitous expression of MSTN in crustaceans was similar to the previous reports in vertebrate systems and other TGF-β family members. The MSTN expression (GDF 11) was observed in both muscular and non-muscular tissues in *Salmo salar* (Ostbye et al., 2001) and *D. rerio* (Biga et al., 2005) respectively. The presence of MSTN expression in both muscular and non-muscular tissues provides additional support to the multi-functional role played by MSTN gene. Therefore, further studies are required to elucidate the possible functional roles of MSTN gene in the invertebrate system.

To date MSTN expression has been reported to have a vital role in growth regulation in crustaceans. Recently MSTN expression during the different stages of larvae have been reported in *L. vannamei* (Qian et al., 2013) where it concludes that *Lv*MSTN expression varies in different tissues as well as at different life stages during the larval development. For some time, the functional roles of MSTN in crustaceans have been

anticipated based on the mRNA transcript at the different molting stages, since crustaceans are known to have discontinuous growth and undergoes molting (Covi et al., 2010; Kim et al., 2009; Kim et al., 2010; MacLea et al., 2010; Qian et al., 2013). More specifically, MSTN in crustaceans have been thought to regulate protein turnover during the molting process (MacLea et al., 2010); to regulate expression in the muscle tissue (Covi et al., 2008); and to serve as a negative regulator by suppressing MSTN protein turnover in the claw muscle (Covi et al., 2010). On the other hand, MSTN of *P. monodon* (De Santis et al., 2011) and *L. vannamei* (Qian et al., 2013) have conversely reported to aid muscle growth.

Such inconsistencies in our understanding of the physiological function of invertebrate MSTN, call for a more precise elucidation of its role. The present study has attempted to achieve this by profiling its expression in *M. rosenbergii* at different fundamental life stages, as well as when the prawn is challenged with IHHNV rather than the previously reported MSTN regulation during molting stages. This should help assist the scientists and breeders in future to target the right life stages to manipulate *Mr*MSTN in order to increase the growth; and also to alter MSTN gene expression in order to combat growth deficiencies caused by IHHNV infection. The result of the *Mr*MSTN mRNA transcription undertaken in this study suggests that the preliminary role of *Mr*MSTN is to serve as a negative growth regulator. This negative regulation upon its presence could in turn slow the muscle development or affect the growth of the prawn.

During the larval stage, the development of *M. rosenbergii* is known to focus on physical changes, as it undergoes approximately eleven zoetal stages (Zoea I-XI) which takes twenty to thirty-five days before developing into the post-larvae (Ling, 1969; Nandlal & Pickering, 2005). On the other hand, extensive muscle development has been

observed during the post-larval stage ranging to the juvenile stages. In consistency with this, *Mr*MSTN expression was observed to be high in the larval stage but then to decrease drastically in the post-larval stage (Figure 4.2). Prawns at the adult stage are mainly focused on reproduction as it is documented that the female adults have a higher tendency to favour reproductive activities over growth and development (MacLea et al., 2010). The activities are concentrated more in the development and maturation of the reproductive organs in order to secure species survival. In line with this, a slight decline in the *Mr*MSTN was observed at the adult stage compared to the juvenile stage.

Previous studies have shown that the larvae and juvenile stages in crustaceans and fish demand high levels of phospholipids supplementation, with larvae consuming more than juveniles (Coutteau *et al*., 1997). Moreover, findings have reported that MSTN expression is correlated with the intramuscular adipose accretion in a Japanese black cattle specifically at the 16^{th} month (Fukuhara, Yamazaki, Nishino, & Tsuchiya, 1970; Shibata et al., 2006). In addition MSTN deletion in mice is reported to decrease the fat accumulation with increasing age (McPherron & Lee, 2002). Based on the available literature and findings in vertebrates, it does seem likely that an increase in the *Mr*MSTN transcript at the juvenile stage can be correlated with a similar adipogenesis process. However, more information on fat composition and the correlation between fat metabolism related gene expression and *Mr*MSTN expression is vital to substantiate this hypothesis in invertebrates.

As we are concerned about the growth of *M.rosenbergii*, in this study we were interested in profiling *Mr*MSTN expression upon infection by the growth affecting virus, IHHNV. Moreover, the stunting of growth upon IHHNV infection leads to seek for a molecular based evidence of the correlation between IHHNV infection and a growth

regulating gene, *Mr*MSTN. The findings of this study could provide a fundamental platform for further research to overcome the growth deficiency. In addition, the preliminary data reporting *Mr*MSTN regulation upon IHHNV infection highlights the importance of the MSTN gene in growth regulation.

The significant increase in expression observed at the sixth hour post injection suggests that *Mr*MSTN does play a role in decreasing muscle mass upon IHHNV infection. This could lead to a runt deformity syndrome in the longer term albeit no distinct changes in the physical appearance were visible over the time period observed during the IHHNV challenge test. MSTN has been reported to be elevated in cases of infection such as Acquired Immunodeficiency Syndrome (AIDS) in *Homo sapiens* (Gonzalez-Cadavid et al., 1998) and hind limb unloading treatment in mice (Wehling et al., 2000), in which both events contribute to muscle mass reduction. Moreover, many researches have postulated MSTN as a therapeutic agent to treat muscle wasting consequence from DMD (Bogdanovich et al., 2002; Bogdanovich, Perkins, Krag, Whittemore, & Khurana, 2005; Wagner et al., 2002). Thus, this preliminary study aids in developing future studies on MSTN as a therapeutic agent for any growth affecting diseases in the aquaculture.

4.6 CONCLUSION

In conclusion, the regulation of *Mr*MSTN transcription at different life stages and upon IHHNV infection suggests the preliminary role of *Mr*MSTN to serve as a negative growth regulator as similar to the vertebrates, therefore it influences the growth and development. Besides that the corresponding *Mr*MSTN regulation upon IHHNV infection suggests that it could influence the growth reduction in the long-term. Thus *Mr*MSTN can be developed as a biomarker and as a potential target for gene manipulation to aid aqua culturists in spotting the infected prawns and to improvise the growth respectively. However, further research is needed on studies of gene knockout, physiological changes and myofibrillar protein contents with support from the current regulation study on *Mr*MSTN in order to determine its role more accurately.

CHAPTER 5

In vivo GENE SILENCING OF *Mr*MSTN: HISTOLOGICAL CHANGES IN MUSCLE TISSUE AND DOWNSTREAM EFFECT ON OTHER GROWTH REGULATING GENES IN *Macrobrachium rosenbergii*

5.1 INTRODUCTION

Gene silencing is an appropriate molecular approach which could help elucidate the distinct role of a gene. In addition gene silencing has been very useful in elucidating the biochemical pathway of a gene as well as its influence on the molecular system. There have been many delivery methods developed in the attempt to knock-down a gene which include double-stranded RNA (dsRNA), small interference RNA (siRNA), micro RNA (miRNA) and short hairpin RNA (shRNA) (Kumar, Singh, Kumari, & Mitra, 2014; Terova, Rimoldi, Bernardini, & Saroglia, 2013; Tripathi et al., 2013; Zhong et al., 2014). The dsRNA silencing was first introduced in *Caenorhabditis elegans* and has been proven of its high efficacy in the target-specific gene knock-down (Fire et al., 1998). Following that the dsRNA silencing has been widely exploited in *Drosophila melanogaster*, planaria, mice and zebrafish (Estrada et al., 2007). In addition, the dsRNA mediated gene silencing has been also successful *in vitro* and *in vivo* especially in crustaceans (Sagi et al., 2013).

Earlier, the succeeding discovery of myostatin (MSTN) gene in vertebrates carrying an interesting role as a negative growth regulator has led to the subject of extensive study specifically in the agriculture and aquaculture sectors. The promising 'double-muscle' mass effect following a gene disruption or natural mutation in animals including mice, in Belgian Blue Cattle Breed (BCBB) and in Piedmontese (Kambadur et

al., 1997; McPherron & Lee, 1997) has been hypothesized to oversee such an ideal increase in the growth production of an economically valued species. Thus, gene silencing was extensively manipulated to elucidate the functional role as well as the efficiency in increasing the muscle mass in goat (Jain et al., 2010), sheep (Hu et al., 2013), chicken (Tripathi et al., 2012) and porcines (Huang et al., 2014). Hence, this potent technique could be a promising tool to further elucidate the distinct role of MSTN in *Macrobrachium rosenbergii* (*Mr*MSTN), representing the invertebrates. Moreover, the finding could also provide substantial support to the preliminary role of *Mr*MSTN as suggested in Chapter 4.

In summary, this study seeks to establish the preliminary histological evidence of the *in vivo* dsRNA silencing of *Mr*MSTN in *M. rosenbergii* and also of its subsequent downstream effects on the regulation of other genes responsible for growth and development in transgenic prawn. We hope that our analysis will give a clearer picture of the role of MSTN in *M. rosenbergii*, and its possible influence on and correlation with other genes of interest for growth and development. Our study could be a pioneer in working with the role of MSTN in invertebrates more precisely. Moreover, this information could provide a fundamental leap in the aquaculture industry towards uplifting the growth production of *M. rosenbergii*.

5.2 LITERATURE REVIEW

The silencing of a MSTN gene will ease in establishing the fundamental phenomena underlying the skeletal muscle growth and hypertrophy (Gabriel, 2009). MSTN gene interruption in the mice has reported a drastic increase in the muscle mass thus confirming its role as a negative regulator in vertebrates (McPherron et al., 1997). Following that, the MSTN has been actively silenced in various vertebrates as well as in different life stages either by *in vitro* or *in vivo* to elucidate its possible functional role (Acosta et al., 2005; Hu et al., 2013; Steelman et al., 2006; Tripathi et al., 2013).

On the other hand, MSTN knockdown in invertebrates particularly crustaceans has poorly explored hence an extensive study is needed to elucidate the precise role of MSTN which still remains ambiguous. Currently the dsRNA silencing of MSTN has been reported in *P. monodon* and *L. vannamei* to serve as a growth favoring gene (De Santis et al., 2011; Lee et al., 2015). This has contradicted with its role as a growth inhibitor in other crustaceans by means of gene expression modulations specifically during molting stages (Covi et al., 2010; Kim et al., 2010; MacLea et al., 2010). Thus following the gene silencing, there is an urge for a detailed analysis to characterize the MSTN role precisely in invertebrates. Therefore, the dsRNA gene silencing technique has been adapted in this study to elucidate the role of MSTN in *M. rosenbergii* and to further be supported by histological analysis.

The histological analysis performed on the tissue of the dsRNA challenged transgenic model enables a transparent view of the possible changes taking place following the gene silencing. A transgenic mice challenged with ATCOL-mediated local administration of MSTN -targeting siRNA shows an increase in the myofibril sizes

compared to the muscles treated with control siRNA/ATCOL (Kawakami et al., 2013). The similar histological findings with a larger tissue were also reported in transgenic sheep with silenced MSTN (Hu et al., 2013). On the other hand, the proliferation and differentiation of the C_2C_{12} myoblast has been disrupted in an increased concentration of a recombinant myostatin *in vitro* and has been proven histologically (Langley et al., 2002).

In addition, MSTN is reported to play diverse roles since it participates in the clustering of the MSTN gene with GDF 11 (a member of the TGF-β family) and in ubiquitous expression in both muscle and non-muscle tissues of vertebrates (Biga et al., 2005; Ostbye et al., 2001) and invertebrates (Covi et al., 2008; Kim et al., 2009; Kim et al., 2010). Moreover previous studies have shown that MSTN is regulated as an effect of alteration of other functional gene expressions wherein the peroxisome proliferator-activated receptor β/ð (PPAR β/ð) (Bonala et al., 2012), ecdysteroid (Covi et al., 2010) and acyl CoA desaturase (Ntambi & Miyazaki, 2003) are altered. This points to the need for further investigation of downstream effects of the silenced *Mr*MSTN gene on other genes of interest for growth and development, particularly those in the growth, reproduction, immunity and fat regulation functional groups. The mRNA regulation of seven growth regulating genes belonging to those groups including myosin heavy chain, dystrophin-dystroglycan complex, tropomyosin, farnesoic acid o methyl transferase, arginine kinase, cyclophilin, and acyl CoA desaturase were profiled under the influence of *Mr*MSTN down-regulation. These potential candidate genes of interest for growth and muscle development in the study, were listed as in Table 2.2 and have already been discussed earlier in Chapter 2.

5.3 MATERIAL AND METHODS

5.3.1 Primer Designing

Primers were designed for the dsRNA synthesis of *Mr*MSTN and GFP from a sequence constructed and obtained from the data respectively and has listed in Table 5.1. The T7 primers were designed by incorporating the region, 5'-TAA TAC GAC TCA CTA TAG GG-3' into both the forward and reverse primers of *Mr*MSTN and GFP respectively. The primers for the co-gene regulation study listed in Table 5.1 were also designed using unigenes obtained from the transcriptome data of *M. rosenbergii* (Mohd-Shamsudin et al., 2013).

5.3.2 RNA Purification and cDNA Synthesis

Total RNA was isolated from *M. rosenbergii* using the TRIZOL Reagent following the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). Total RNA was treated with an RNase free DNA set (5 Prime GmbH, Hamburg, Germany) to remove any contaminating DNA. The total RNA concentration was measured spectrophometrically at OD 260/280 and 260/230 (NanoVue Plus Spectrophotometer, GE Healthcare UK Ltd, England). The first-strand cDNA was synthesized from total RNA by M-MLV reverse transcriptase (Promega, Madison, WI, USA) followed by oligo-dT (20) VN primer in accordance with the manufacturer's protocol.

Table 5.1. List of primers used in the dsRNA silencing and mRNA transcription of growth related genes.

Primer Name	Forward	Reverse	Application
***Mr*MSTN**	TAGTAGCACCCAAGGTCTACGA	TAAGAGCACCCACAACGATCCA	Silencing/ qRT-PCR
T7-*Mr*MSTN	TAATACGACTCACTATAG TAGTAGCACCCAAGGTCTACGA	TAA TAC GAC TCA CTA TAG TAAGAGCACCCACAACGATCCA	Silencing
GFP	ATG GTG AGC AAG GGC GAG GA	TTA CTT GTA CAG CTC GTC CA	Silencing/ qRT-PCR
T7-GFP	TAA TAC GAC TCA CTA TAG GAT GGT GAG CAA GGG CGA GGA	TAA TAC GAC TCA CTA TAG GTT ACT TGT ACA GCT CGT CCA	Silencing
Myosin Heavy Chain	GACCTCGTCTGCCAAGTGAA	TTTGTCACGTAGCGAGCCTT	qRT-PCR
Dystrophin-dystroglycan	ACAGATACATGTTCCGGCTAAT	CCAAATGCTGCAACCTCAC	qRT-PCR
Tropomyosin	GAAGGACAACGCCATGGACA	TCTGCGTCGATCGAATCTCC	qRT-PCR
Acyl Co-A Desaturase	AGGCCACAACTGGGTTTTGT	CGGTACATGAGCCATCCCAT	qRT-PCR
Farnesoic acid O-Methyl transferase	GGTGGCTGGGATAACCAGAA	CTTCCTTTCCTCGTCACAACA	qRT-PCR
Arginine Kinase	AAGATCAAGGGCAACATCAACGCC	AGGCGCTCTTCATGAGGTTCTCAA	qRT-PCR
Cyclophilin	TTTCCCATGACGCCTTTCGT	CATACGGCGACTGCTACCTC	qRT-PCR

5.3.3 Cloning of *Mr*MSTN cDNA

PCR amplification for *Mr*MSTN and GFP were carried out using respective primer (Table 5.1) in the following conditions : 1.5 µM $MgCl_2$, 5 µM 10X Buffer, 10 µM dNTPs, 10 µM forward and reverse primer, (1.25 U/25 µL) Go Taq Polymerase and 50ng of cDNA in a total reaction volume of 10 µL. The PCR profile for *Mr*MSTN was as follows: initial denaturation 94 °C for 5 min, 28 cycles of 94 °C for 30 s, annealing temperature 55.3 °C for 30 s, and 72 °C for 30 s, final extension at 72 °C for 7 min. The PCR profile for GFP was as follows: 94 °C for 2 min, 28 cycles of 94 °C for 30 s, annealing temperature 55.0 °C for 30 s, and 72 °C for 30 s, final extension at 72 °C for 10 min. PCR products were separated with 1.5% agarose gel electrophoresis and identified on the UV transilluminator after staining with ethidium bromide. The PCR products with expected sizes were purified using a Gel Extraction Kit (Geneall, Seoul, Korea), ligated into the TA plasmid vector using the pGEM-T Easy Cloning Kit (Promega, Madison,WI, USA) and transformed into a One Shot Top 10 Escherichia coli. The isolated plasmid was then used as a template to perform PCR and degenerate PCR for *Mr*MSTN and GFP using the same profile respectively but 18 cycles for the later one. The end product then purified for further dsRNA – *Mr*MSTN and dsRNA – GFP synthesis as described in section 5.3.4. The primers designed for PCR synthesis of *Mr*MSTN and GFP were displayed in Table 5.1. Nucleotide sequences of the cloned cDNAs were determined using an automated genetic analyzer (ABI Biosystem, Carlsbad, CA, USA).

5.3.4 Double-stranded RNA (dsRNA) Molecule Synthesis

The primers designed for the dsRNA synthesis of *Mr*MSTN and GFP are listed in Table 5.1. Double-stranded RNA (dsRNA) synthesis and microinjection were performed as recommended by Manningas et al. (2008). Briefly, dsRNA was produced using T7 RiboMAX large scale RNA production system in accordance with the manufacturer's protocol (Promega, Madison, WI, USA). The pGEM Express Positive Control were provided which produces transcripts that are 1.1kb and 2.3kb in length. The previously isolated plasmid was then used as a template to perform PCR and degenerate PCR for *Mr*MSTN and GFP using the same profile respectively but with 18 cycles for the later one. Two different PCR sets for each gene containing T7-Forward - Reverse primer pair and Forward - T7-Reverse primer pair were generated.

Two separate sets of single stranded RNA (ssRNA) were then synthesized in the following conditions : 3 µg purified degenerated PCR products, 10 µL Ribomax express T7 2X buffer, 2 µL Enzyme mix T7 express and Nuclease free water in a total reaction volume of 20 µL. The mixture was gently mixed and incubated at 37 ^{0}C for 30 m. Following that, equal amounts of ssRNA from both sets were annealed by incubating at 70 ^{0}C for 10 m and then slowly cooled at room temperature to convert it into dsRNA. The resulting dsRNA (5 µg) was purified and stored at -80 ^{0}C for further *in vivo* experiments.

5.3.5 Microinjection into *Macrobrachium rosenbergii*

M. rosenbergii (approximately 8 g) were obtained from a local prawn farm at Negeri Sembilan, Malaysia. The prawns were handled with care as per the animal ethical procedure which is described here. Formal approval from the related authorities of the

university had been taken into account conduct the study, thus enabling the prawn farm owner to supply the prawns. The prawns were transported to the laboratory in oxygenated polythene bags. In the laboratory, the prawns were acclimatized over a period of one a week. The prawns were reared in 300 L flat-bottom glass tanks by supplying additional aeration and de-chlorinated freshwater. During acclimatization, the water was changed daily and the best water quality conditions (pH 7.1 and Temperature 28 ± 2 °C) were maintained as suggested by Buikema et al (1982). Moreover, during acclimatization, the prawns were fed twice a day at 0900 h and 1600 h with commercial prawn pellets. This work was exempted from official approval from the Institutional Animal Care and Use Committee as the prawns are regarded as edible shellfish.

Five µg of dsRNA molecule of dsRNA-*Mr*MSTN and dsRNA-GFP were injected intramuscularly into the prawns and the samples were collected at 7^{th} day post-injection (d.p.i) and 14^{th} d.p.i for further processing. The prawns were re-injected with the assigned treatment on day 7 for the 14^{th} d.p.i of the experiment. For each time point of microinjection, muscles were collected from four replicates and snap-frozen in liquid nitrogen, followed by storage at -80°C for further qRT- PCR analysis. The dsRNA-GFP and non-challenged prawns served as positive and negative controls respectively.

5.3.6 Histological Analysis

Histology preparation of specimens for normal histology was done accordingly (Bell & Lightner, 1984). The experimental challenge group of prawns (n=4) include the dsRNA-*Mr*MSTN, dsRNA-GFP and non-challenged dsRNA groups for both 7^{th} d.p.i and 14^{th} d.p.i. All the experimental prawns were injected with Davidson's fixative before fixing

in Davidson's solution for 24 – 48 hours. The samples were then transferred to 70% alcohol for subsequent histo-slide preparation as stated below in sub-section.

5.3.6.1 Tissue processing and embedding

Muscle samples of all the experimental prawns were processed with a series of alcohol washing in the following order; 70% Ethyl Alcohol (EtOH) (two separate one hour baths), 80% EtOH (two separate one hour baths), 95% EtOH (two separate one hour baths), 100% EtOH (two separate one hour baths), Clearing agent - Xylene (two separate one hour baths), and paraffin (two separate one hour baths). The tissues were then embedded in a hard paraffin block, which was then placed in a mold containing excess molten wax and was allowed to cool and harden in open air space. The embedded cassettes containing the samples were then transferred to the cassette bath which was pre-filled with molten wax.

5.3.6.2 Sectioning

The sectioning was done using the AO rotary microtome 820 (American Optical, Scientific Instrument Division Buffalo, New York). The paraffin block with the embedded muscle tissue is placed in the microtome holder and its level adjusted to the edge of knife for further trimming process. The trimming was done with the thickness gauge at 10-15 micrometre (µm) initially, and followed by 5 µm as the cutting appears to be even. The thickness section of four micrometers was gently teased off with the aid of a fine hair brush and forceps. The samples were then placed on the slides with the aid of a few water drops and the sample identity (ID) was written at the edge of the glass slides

with a sharp point crafting pen. The slides were then placed in the oven for two hours or overnight in an incubator with the oven set to 40 ^{0}C.

5.3.6.3 Staining With Hematoxylin and Eosin (H&E)

The samples obtained from the above protocol were then stained by the Hematoxylin and Eosin (H&E) procedure (Sheehan et al., 1980) and subsequently mounted with DPX (mixture of distyrene, a plasticizer, and xylene) and covered with cover slip. The slides were then placed in an incubator oven for two hours or overnight set at 40 ^{0}C. The staining procedure has been illustrated in Appendix C. Finally the slides were all set for examination under a Leica DM 5000B (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) compound microscope which was connected to a Leica DFC 320 (Cambridge, United Kingdom) digital camera that associated with a computer software, Leica Q Win (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). The density of the distributed nuclei cells was performed according to (McCann, Ozolek, Castro, Parvin, & Kovacˇevic´, 2015) and one way anova was performed using SPSS to validate the results obtained .

5.3.7 Quantitative Real-Time PCR Analysis of *Mr*MSTN and Other Genes of Interest for Growth and Development in dsRNA Challenged *M. rosenbergii*

A quantitative real-time PCR analysis (qRT-PCR) was carried out using an ABI 7500 Real-time Detection System (Applied Biosystems) in a 20 µL reaction volume containing 50 ng of cDNA from each tissue, Power SYBR_ Green Master Mix, 0.3 µM of each primer and 7.8 µL dH2O. The qRT-PCR cycle profile was 1 cycle of 95 ^{0}C for 10 m, followed by 40 cycles of 95 ^{0}C for 10 s and 60 ^{0}C for 1 m. The same qRT-PCR cycle

profile was used for GFP, other selected growth related genes and the internal control gene, EF-1. The primers used in this study are presented in Table 5.1. EF-1 (Elongation Factor – 1) primers were designed based on an EST similar to the elongation factor 1-alpha from *M. rosenbergii* (GenBank accession No. EL609261). After the PCR program, the data was analyzed with ABI 7500 SDS software (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. The comparative CT method (2^{-ddCT} method) was used to analyse the expression level of *Mr*MSTN (Livak & Schmittgen, 2001). All data were expressed in terms of means ± standard deviation of the relative mRNA. To compare relative mRNA expression of *Mr*MSTN and other growth regulating genes, statistical analysis was performed using a one-way ANOVA, and the mean comparisons were performed by Tukey's Multiple Range Test using SPSS 11.5 at a 5% significance level.

5.4 RESULTS

5.4.1 Synthesis of Double-stranded RNA (dsRNA) Molecule

Table 5.1 shows the primers designed for dsRNA synthesis. The dsRNA-*Mr*MSTN and dsRNA-GFP were generated by annealing two sets of T7-Forward and T7-Reverse ssRNA together. The ssRNA sets were produced from the degenerate PCR products which used the plasmid as the template. Figure 5.1 shows the synthesized dsRNA molecules of *Mr*MSTN and GFP respectively.

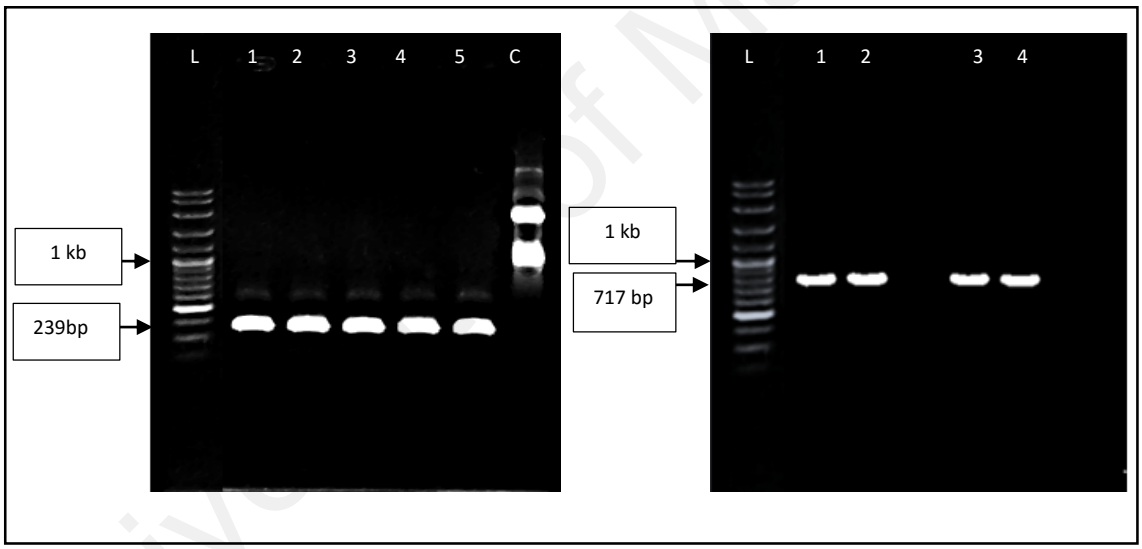


Figure 5.1. Figure represents 1% (w/v) agarose gel running of synthesized double-stranded RNA (dsRNA) :- (A) dsRNA-*Mr*MSTN molecule. Lane 1-5: dsRNA – *Mr*MSTN, Lane L: Vivantis 100bp ladder, Lane C: pGEM express positive control (Promega). (B) dsRNA-GFP molecule. Lane 1-4: dsRNA – GFP, Lane L: Vivantis 100bp ladder.

5.4.2 *Mr*MSTN mRNA Transcription in dsRNA-*Mr*MSTN Challenged *M. rosenbergii*

The synthesized dsRNA molecules (5 µg) of *Mr*MSTN and GFP were injected into prawns weighing approximately 8 g (n = 4) and the samples of prawn (muscle) from the 7th d.p.i and 14th d.p.i were collected. The Figure 5.2 shows that the relative expression of *Mr*MSTN was significantly down regulated in the dsRNA-*Mr*MSTN injected prawn, compared to the dsRNA-GFP (positive control) and unchallenged prawn (negative control) in both the experimental periods. The results also ensures the specificity of dsRNA as the *Mr*MSTN mRNA was down regulated in dsRNA-*Mr*MSTN challenged prawn but independently expressed in dsRNA-GFP challenged prawn.

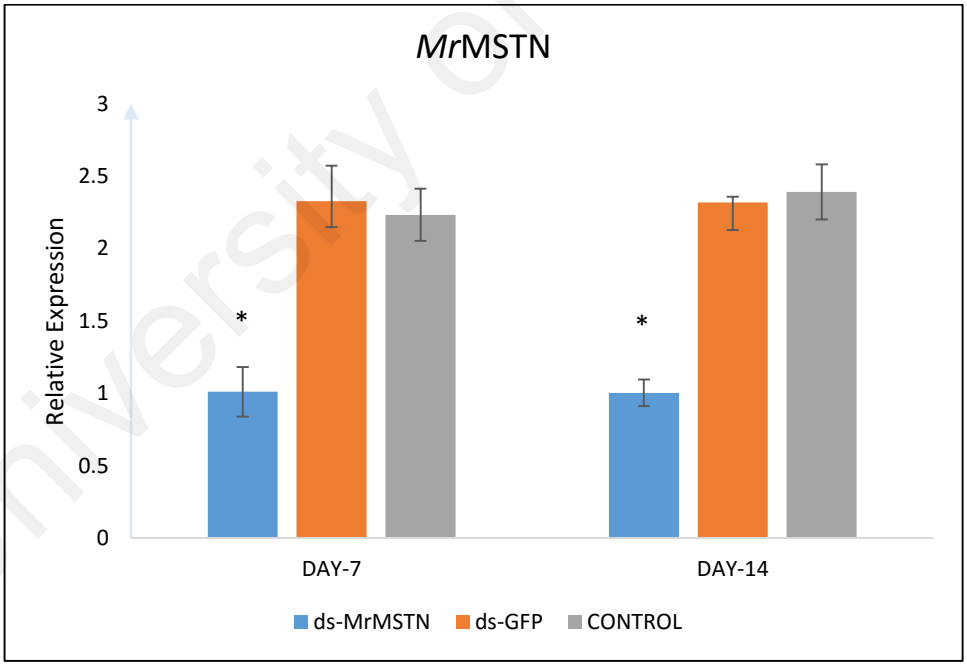


Figure 5.2. Graph representing relative expression of *Mr*MSTN in *M. rosenbergii* (muscle) injected with dsRNA-*Mr*MSTN, dsRNA-GFP (positive control) and uninjected prawn (negative control) at 7th d.p.i (Day-7) and 14th d.p.i (Day-14). *Mr*_EF (expressing at similar levels in all tissues) served as the constitutively expressed control. The statistical analysis was based on the comparison of the relative expression ratio of *Mr*MSTN gene to the *Mr*_EF by calculating $2^{-\Delta\Delta Ct}$ values. Data are expressed as a ratio of *Mr*MSTN mRNA lowest expression. The significant differences in *Mr*MSTN expression ($P < 0.05$) between the challenged and the control group are indicated with asterisks (n=4).

5.4.3 Histological Analysis

Figure 5.3 and 5.4 shows the H&E stained muscle tissue of dsRNA-*Mr*MSTN challenged, dsRNA-GFP challenged and non-challenged *M. rosenbergii* on 7th d.p.i and 14th d.p.i respectively. The histological analysis shows H&E stained nuclei in abundance within dsRNA-*Mr*MSTN challenged muscle when compared to the controls in *M. rosenbergii* on 7th d.p.i and 14th d.p.i. Moreover, this significant increase in the nuclei cells has been proven statistically by nuclei density calculations (Figure 5.5). The density was calculated as the number of nuclei cells over size, 80 μm^2 and one way anova was performed to validate the result obtained statistically. The area size of 80 μm^2 was determined using ImageJ software. In addition, a remarkable fiber with the presence of a central nuclei as well as fusion of a number of nuclei cells were observed on 14th d.p.i.

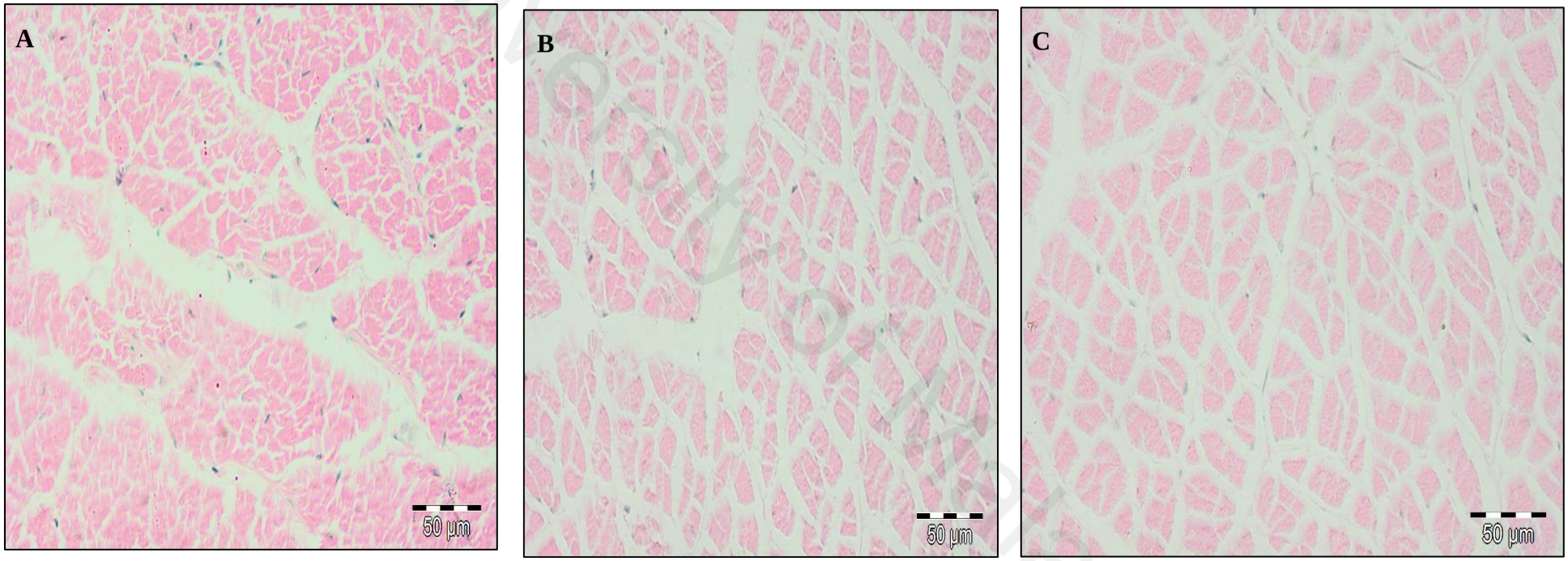


Figure 5.3. 20-X microscopic observation of H& E histological staining of muscle tissue of *M. rosenbergii* (n=4) on 7th day post injection *(d.p.i)*. A: dsRNA-*Mr*MSTN challenged muscle. B: dsRNA-GFP challenged muscle. C: Untreated sample. Bar scale: 50 µm

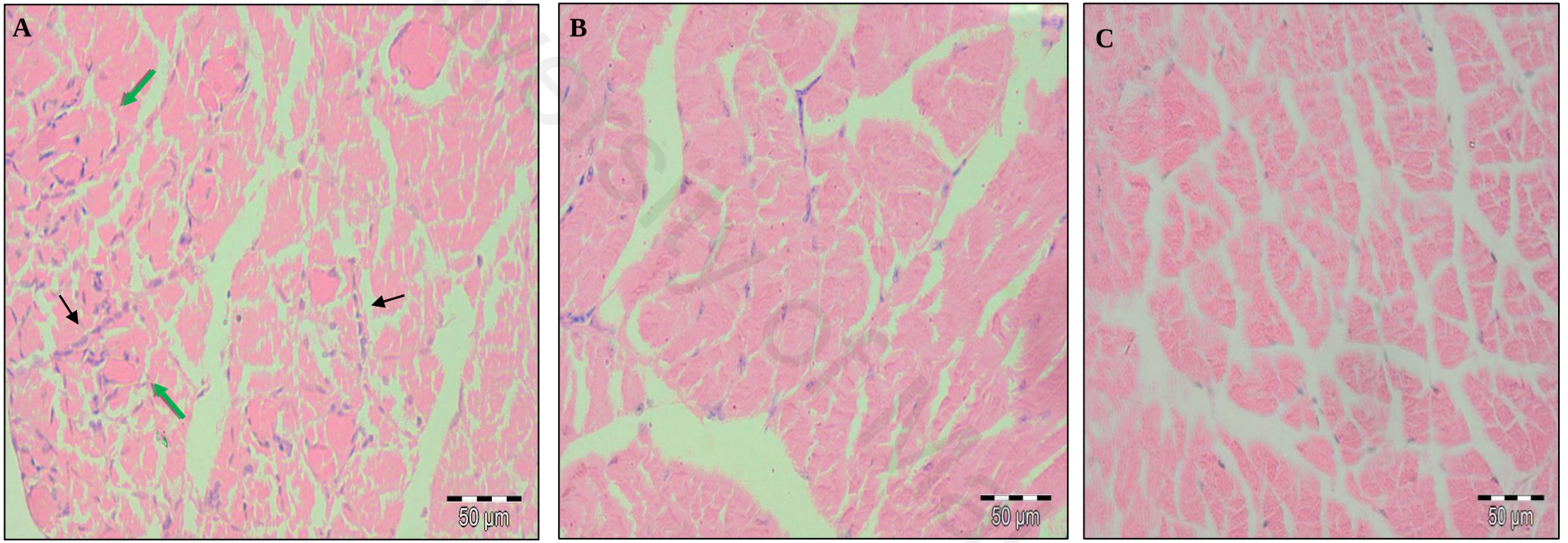


Figure 5.4. 20-X microscopic observation of H& E histological staining of muscle tissue of *M. rosenbergii* (n=4) on 14th day post injection (d.p.i). A: dsRNA-*Mr*MSTN challenged muscle. B: dsRNA-GFP challenged muscle. C: Untreated sample. Arrow indication on dsRNA-*Mr*MSTN challenged muscle tissue (A): Green- fiber with central nuclei; Black – Fusion of number of nuclei cells. Bar scale: 50 µm.

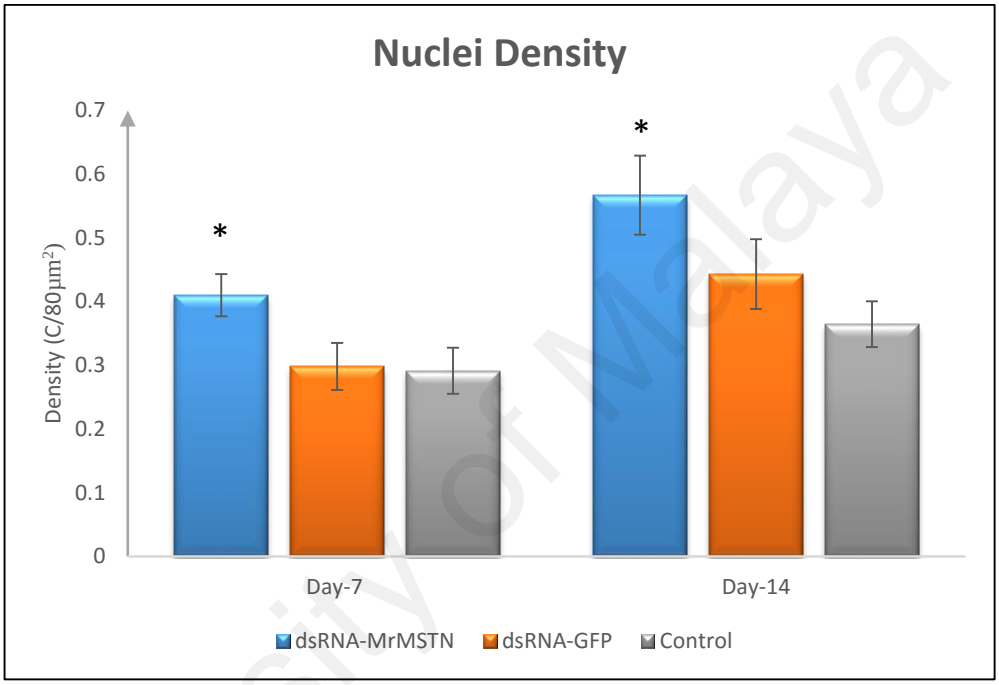


Figure 5.5. Graph represents the density of nuclei cell in dsRNA-*Mr*MSTN challenged study in *M. rosenbergii* (muscle) on 7th d.p.i (Day-7) and 14th d.p.i (Day-14). The statistical analysis was based on the comparison of the density of dsRNA-*Mr*MSTN challenge with the dsRNA-GFP challenged and unchallenged *M. rosenbergii*. The significant increase ($P< 0.05$) in the density of dsRNA-*Mr*MSTN challenge is indicated with asterisks.

5.4.4 mRNA Transcription of Selected Growth Regulating Genes in dsRNA-*Mr*MSTN Challenged Sample

Overall, there were some alterations in the regulation of the selected growth and development related genes on 7th d.p.i and 14th d.p.i of dsRNA-*Mr*MSTN challenged muscle tissue (Figures 5.6). The mRNA transcripts of the myosin heavy chain, dystrophin-dystroglycan, cyclophilin and acyl co-A desaturase showed signs of down-regulation upon *Mr*MSTN down-regulation. Meanwhile, the tropomyosin showed progressive up-regulation, whereas the farnesoic acid o-methyl transferase and arginine kinase showed up-regulation on 7th d.p.i and 14th d.p.i respectively.

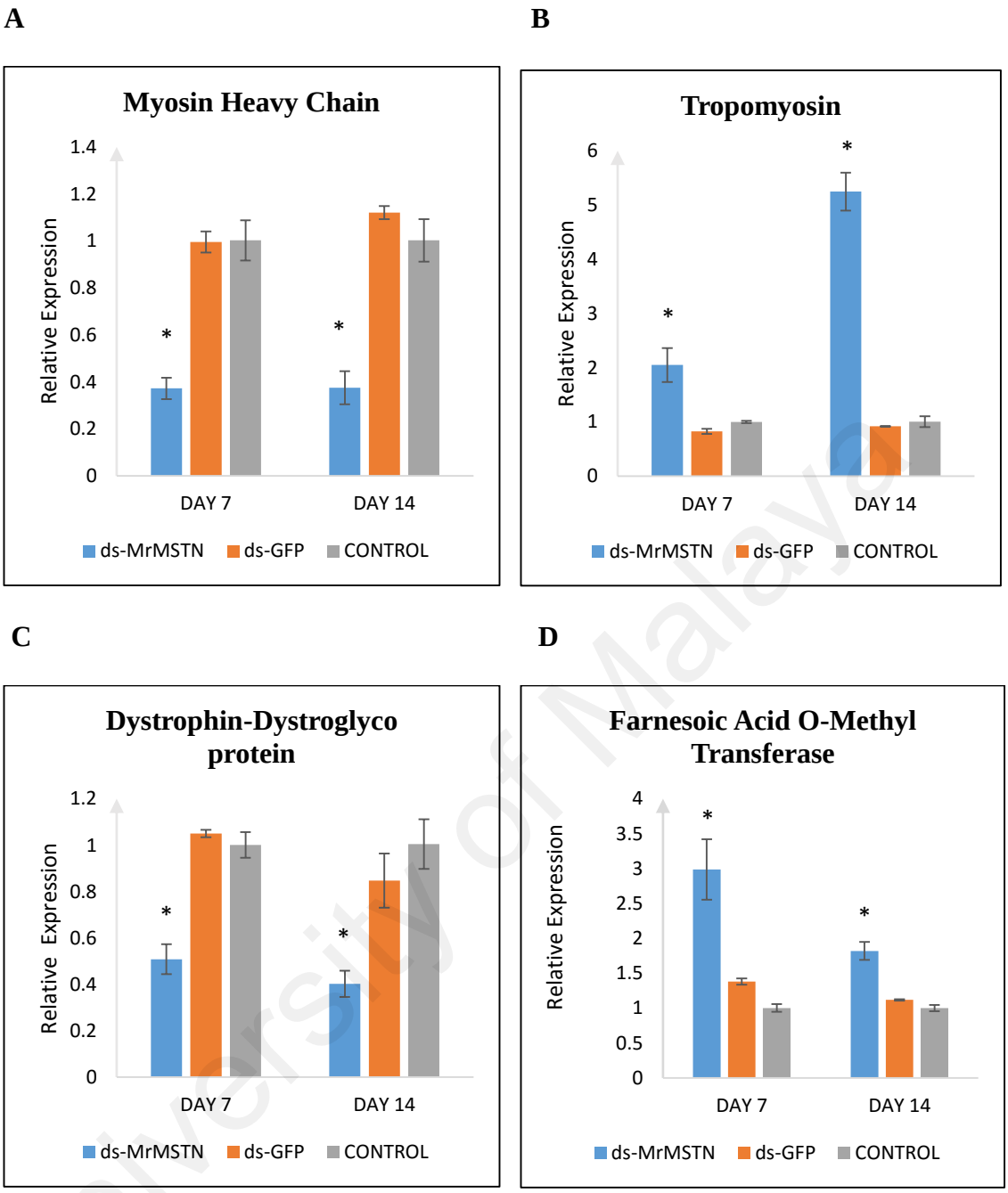
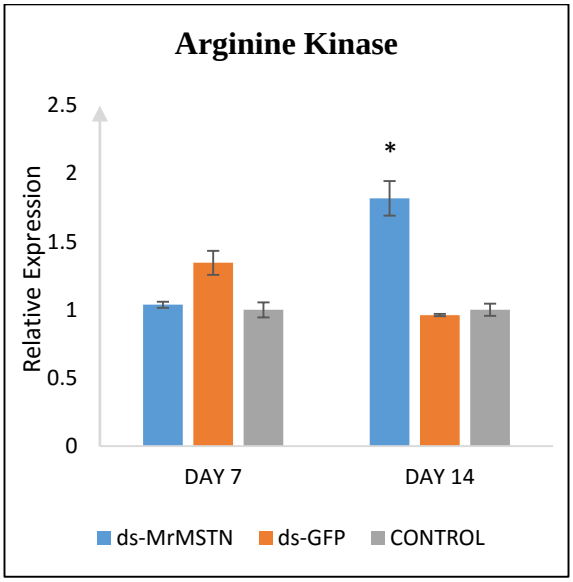
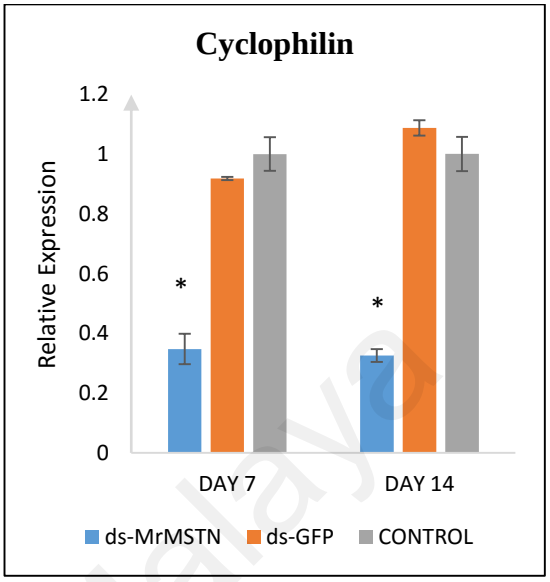


Figure 5.6. Graphical representation showing the mRNA transcripts of genes of interest for growth and development following dsRNA-*Mr*MSTN challenged in *M. rosenbergii* (muscle) for the time interval of 7th d.p.i (Day 7) and 14th d.p.i (Day 14): A-Myosin Heavy Chain, B-Tropomyosin, C-Dystrophin, D-Farneosic Acid O Methyl Transferase. The statistical analysis was based on comparison of the relative expression ratio of *Mr*MSTN gene to the *Mr*_EF by calculating $2^{-\Delta\Delta Ct}$ values. Data are expressed as a ratio of the mRNA expression of negative control (CONTROL). The significant differences in *Mr*MSTN expression ($P< 0.05$) between the challenged and the control groups are indicated with asterisks (n=4).

E



F



G

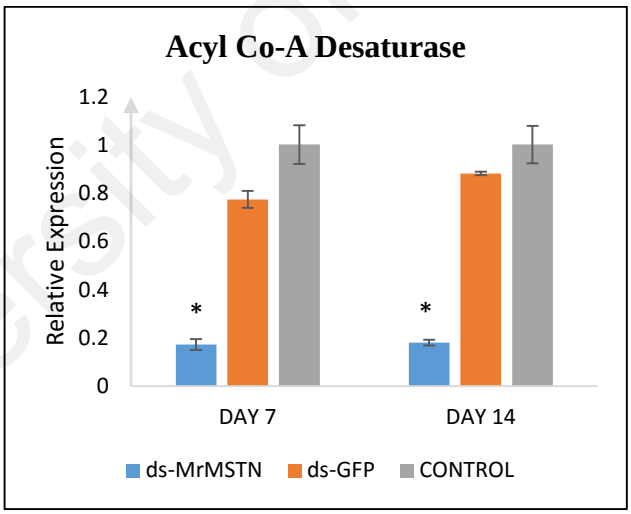


Figure 5.6 (Continued). Graphical representation showing the mRNA transcripts of genes of interest for growth and development following dsRNA-*Mr*MSTN challenged in *M.rosenbergii* (muscle) for the time interval of 7th d.p.i (Day 7) and 14th d.p.i (Day 14): E-Acyl Co-A Desaturase, F-Arginine Kinase, and G-Cyclophilin. The statistical analysis was based on comparison of the relative expression ratio of *Mr*MSTN gene to the *Mr*_EF by calculating $2^{-\Delta\Delta Ct}$ values. Data are expressed as a ratio of mRNA expression of negative control (CONTROL). The significant differences in *Mr*MSTN expression ($P< 0.05$) between the challenged and the control group are indicated with asterisks (n=4).

5.5 DISCUSSION

The dsRNA-*Mr*MSTN mediated *in vivo* study conducted at fixed time points that are on 7th d.p.i and 14th d.p.i shows that *Mr*MSTN gene has been successfully down-regulated in *M. rosenbergii*. Following the dsRNA-*Mr*MSTN challenge, the histological changes in the muscle tissue and its downstream effect on other genes of interest for growth and development have also been examined in this study and discussed below.

5.5.1 dsRNA-*Mr*MSTN *in vivo* Gene Silencing in *M. rosenbergii*

Silencing is a good mediator tool for understanding the functional role of genes. It has been gradually gaining ground as a mode of study, especially in crustaceans where the gene manipulation could help boost aquaculture production in near future (Sagi et al., 2013). Fire et al (1998) has initiated dsRNA mediated silencing in the *Caenorhabditis elegans* model, and demonstrated its silencing effect up to the progeny level. Since then, the dsRNA has been used extensively in *in vivo* studies, especially in crustaceans, for its well-known stability and efficiency in silencing transcriptional genes (Estrada et al., 2007). This study similarly seeks to silence the *Mr*MSTN gene as a means of closely examining its distinct role in invertebrates, which is still a topic of debate.

The dsRNA mediated silencing of the MSTN gene, similar to the down-regulation of *Mr*MSTN mRNA transcript has previously been reported in crustacean species including *P. monodon* (De Santis et al., 2011) and *L. vannamei* (Lee et al., 2015). However, the dsRNA silencing of MSTN in *P.monodon* and *L. vannamei* shows a decrease in weight; thus suggesting it to serve as a growth promoter. This has put the overall role of MSTN in invertebrates, specifically crustaceans controversial. The current

study has extended the silencing work, reporting histological changes in the muscle tissue as well as gleaning into its impact on other growth-regulating genes in order to provide a more robust definition to its functional role in growth development specifically on muscle.

5.5.2 Histological Analysis

Following the dsRNA-*Mr*MSTN challenge, the subsequent phenotypic changes in the muscle tissue for the time points of 7^{th} d.p.i and 14^{th} d.p.i has been perceived through histological analysis. The histological analysis provides with a proof that the successfully silenced *Mr*MSTN could probably to trigger the activation of satellite cells which indicates the onset of the regeneration process. The increase in nuclei cell numbers found in this study is consistent with the earlier finding (Schultz & McCormick, 1994) which have shown a similar increase in the cytoplasmic content, towards muscle regeneration upon muscle injury or tension. The presence of new fibers with nuclei in the center as well as the fusion of many nuclei cells on 14^{th} d.p.i in the dsRNA-*Mr*MSTN injected muscle indicates the occurrence of muscle regeneration and hyperplasia. A similar histological pattern was observed in the skeletal muscle responding to a toxin-induced injury hence confirming the muscle regeneration (Hawke & Garry, 2001).

Satellite cell activation is the first step towards a successful myogenesis process. Many findings have shown that vertebrate MSTN can inhibit proliferation and differentiation in the myogenesis process (Joulia et al., 2003; Langley et al., 2002). Initially, TGF-β was reported as one of the growth factors influencing the activation of satellite cells by inhibiting their proliferation and differentiation (Allen & Boxhorn, 1989). Previous studies have reported that in the absence of MSTN, satellite cells were

activated along with an increase in the expression of regeneration markers in senescent mice (Wagner et al., 2005) as well as increase in the total number of satellite cells per unit length of the muscle fiber (McCroskery et al., 2003) . In addition, Dong et al (2013) reported that satellite cells are activated along with the up-regulation of Akirin-1, which indirectly suppress the MSTN gene. Hence, there is a clear consensus that skeletal muscles do not regenerate without satellite cells, confirming their pivotal and non-redundant roles in myogenesis (Relaix & Zammit, 2012). Thus, further work can be performed in future to confirm current increase in nuclei is belongs to the increase in satellite cell upon *Mr*MSTN downregulation.

In general the satellite cell activation is followed by proliferation that takes place within the first week after a muscle injury (Ciciliot & Schiaffino, 2010). Moreover, the histological analysis confirm, that the sequential event of satellite cell activation, proliferation and differentiation has been finally followed by the regeneration of fibers with centrally located nuclei, which has been commonly observed in the prolonged absence of MSTN in vertebrates (Dong, Pan, & Zhang, 2013; Wagner et al., 2005). In addition, the presence of the central nuclei in the fibers are a strong pointer towards the regeneration process, indicating the formation of new fibers (Nicole et al., 2003). In summary, the histological patterns observed in the period following the injection of dsRNA-*Mr*MSTN strongly support the contention that MSTN in crustaceans share the same functions as of a growth inhibitor as observed in vertebrates. However, there is a need for in-depth study, especially in other members of crustaceans to provide additional robust evidence to support the current findings.

5.5.3 dsRNA-*Mr*MSTN Downstream Effect on mRNA Transcript of Selected Genes of Interest for Growth and Regulation

The down-regulation of *Mr*MSTN has shown to alter the regulation of seven genes that are related to growth and development. The possible reason for the gene alteration following the dsRNA-*Mr*MSTN injection has been described below. The mRNA profiling of these seven genes were regulated in response to the *Mr*MSTN down-regulation and subsequent muscle regeneration as explained further below.

5.5.3.1 Myosin Heavy Chain (MyHC)

The MyHC is closely related to muscle contraction and exists with many isoforms where each are ubiquitously expressed in all eukaryotic cells (DeNardi et al., 1993; Jung et al., 2011). However, the expression levels vary depending on the muscle types – i.e. whether it includes plantaris (fast) or/and soleus (slow) muscle (LaFramboise et al., 2000). The MSTN is reported to alter the MyHC specifically through the activin receptor and is influenced by different myofiber types (Wang, Yu, Kim, Bidwell, & Kuang, 2012). In a MSTN knockout mice, the slower (MyHC I and II) and faster MyHC have been reported to decrease and increase respectively compared to the wild type (Gabriel, 2009; Girgenrath et al., 2005).

In crustaceans, an isoform of MyHC has been reported in the embryos of *H. americanus* during the muscle formation examination (Harzsch & Kreissl, 2010). MyHC has been used as a marker to establish myogenesis in crustaceans including a marbled crayfish (Jirikowski, Kreissl, Richter, & Wolff, 2010) as well as two isopods, *Porcellio scaber* and *Idotea balthica* (Kreissl, Uber, & Harzsch, 2008). Moreover, recent

investigation has reported MyHC as a potential target for ecdysteroid control along with other genes which include molting and chitin metabolism related genes (Qian et al., 2014). In accordance with the MyHC findings, similar alteration in MyHC, subsequently with dsRNA-*Mr*MSTN injection suggest that its down- regulation could favour muscle regeneration. This provides further evidence that the MSTN function is as similar to that in vertebrates. However, MyHC have to be further characterized in *M. rosenbergii* to understand the relationship better specifically in myogenesis.

5.5.3.2 Tropomyosin – MEF2

There are only limited findings of tropomyosin mRNA expression in the myogenesis process in the existing crustacean database. Recently, two types of tropomyosins, which play a role in sex determination were characterized in *Macrobrachium nipponense* (Jin et al., 2014). In *Drosophila*, the myocyte-specific enhancer factor 2 (MEF2) highly regulates the expression of tropomyosin especially in the adjoining muscle activation, second cis-acting region during embryonic, larval, and adult myogenesis (Lin et al., 1997; Lin et al., 1996). Moreover, the MEF2 is also shown to regulate adult myogenesis in *D. melanogaster* (Baker, Tanaka, Klitgord, & Cripps, 2005). In addition, the MEF2 is characterized to influence the regulation of many growth related genes including tropomyosin, muscle lim protein and actin directly or indirectly; thus explicating its profound role in controlling myogenesis (Kelly, Meadows, & Cripps, 2002; Lin et al., 1996; Stronach, Renfranz, Lilly, & Beckerle, 1999). Our findings suggest that the up-regulation of tropomyosin-MEF2 along with the down-regulation of MSTN, could favour the myogenesis process, although a detailed study of this is needed. Interestingly, the study observation on a similar increase in allergen contributors, tropomyosin and arginine

kinase on 14th d.p.i is also a fact to be pondered. The MSTN could also contribute to the allergy in crustaceans which should be also be investigated in the near future.

5.5.3.3 Dystrophin- dystroglycan complex

Till this day, DMD has been extensively studied in the vertebrate system. Turning specifically to dystrophin- dystroglycoprotein complex encoding gene, it has been extensively studied in the vertebrate system (Campbell, 1995) and the MSTN gene has been postulated as a potential therapeutic agent to rectify the duchenne muscular dystrophy (DMD) (Bogdanovich et al., 2002; Wagner et al., 2002). However this gene has not been fully characterized in invertebrates, or specifically crustaceans, so its functional role in this animal remains unclear. In zebrafish, the elevated expression of MSTN-2 has been reported to decrease the regulation of dystroglycoprotein complex including the dystroglycan and the sarcoglycan (Amali et al., 2008). In contrary, the down-regulation of MSTN is inversely correlated to the DGC including dystroglycan mRNA which favours the cranial sartorius hypertrophy in a golden retriever muscular dystrophy (GRMD) canine (Nghiem et al., 2013). In addition, a reduction in the dystrophin- dystroglycoprotein complex has been observed in the mdx mouse skeletal muscle, suggesting it might cause alterations in the dystrophin but not in muscle fiber degradation (Ervasti & Campbell, 1993) or muscle necrosis (Ohlendieck, 1996). Thus the reduction in the dystroglycan here need to be studied further of the respective gene functional role and contribution to the anticipated myogenesis.

5.5.3.4 Farnesoic Acid O-Methyltransferase (FAMeT)

FAMeT is an enzyme that catalyzes methyl farnesoate production, which influences reproduction and growth regulation specifically molting in crustaceans (Gunawardene et al., 2002; Hui et al., 2008; Reddy, Nagaraju, & Reddy, 2004). Moreover, its regulation coincides with the regulation of ecdysteroid in the molting process (Nagaraju, 2006; Tamone & Chang, 1993). Conversely, during the molting stages MSTN has been reported to be altered (Covi et al., 2010; Kim et al., 2009; Kim et al., 2010; MacLea et al., 2010) and inversely regulated with the ecdysteroid especially during molting process (Covi et al., 2010). Thus previous findings on the FAMeT with ecdysteroid and MSTN with ecdysteroid supports the current elevated FAMeT in the dsRNA-*Mr*MSTN challenged prawn albeit no direct correlation between MSTN and FAMeT. Moreover, this preliminary finding also suggesting that FAMeT may favour progressive muscle hypertrophy as the MSTN is down-regulated. In addition it also confirms the role of *Mr*MSTN as a negative growth regulator in invertebrate as similar to that earlier reported in vertebrates

5.5.3.5 Arginine Kinase (AK)

In our previous findings we have proved that arginine kinase (AK) (Arockiaraj et al., 2011) and *Mr*MSTN (Sarasvathi, Bhassu, Maningas, & Othman, 2015) are abundant in the muscle tissue of *M. rosenbergii* and are regulated upon infectious hypodermal and hematopoietic necrosis virus (IHHNV), a growth affecting infection. This has prompted us to attempt to elucidate further the impact of *Mr*MSTN on AK, since both are highly expressed in muscle and both respond to similar pathogenic challenges. The upregulated expression of AK on 14^{th} d.p.i of our test suggests a possible additional energy supply,

favoring muscle hyperplasia. Overall, AK's role has variously been depicted as the supply of additional ATP in fatiguing and acidic environments (Ellington, 1989; Walter, Barton, & Sweeney, 2000); binding to actin with a reasonable affinity in the scallop muscle (Reddy, Houmeida, Benyamin, & Roustan, 1992); and the coupling of energy production and immune responses in shrimps (Arockiaraj et al., 2011; Yao et al., 2009). Although AK is clearly abundant in muscle and has a functional role in ATP regulation, the understanding of its relationship in muscle formation especially in extreme conditions such as muscle regeneration or degeneration, muscle related diseases and necrosis is yet to be discovered. Our study is the first to attempt to establish a relationship between AK and a growth-regulating gene, *Mr*MSTN hence paving way for further exploration of the gene's role in growth development. Moreover, this study has also observed the similar higher expression of allergen related genes, AK and tropomyosin on 14th d.p.i suggest *Mr*MSTN could influence the allergy in crustaceans thus should be considered in further studies.

5.5.3.6 Cyclophilin A (CypA)

A recent study, shows that the expression of CypA negatively correlates with body weight, as the mRNA transcript of CypA is lower in larger female shrimp than in smaller ones (Tangprasittipap, 2010). Moreover, CypA is one of the five genes identified as likely to be involved in cell differentiation or proliferation, cell cycle and hormone processing (Tangprasittipap, 2010). Conversely, the expression of cyclophilin along with myosin and haemocyanin is reported to be relatively high within a larger body mass than in lower weight shrimp (Kamimura et al., 2008). The down-regulation of cyclophilin following the dsRNA-*Mr*MSTN challenge is hypothesized to supports the idea that muscle regeneration could be taking place. However, there is as yet no solid evidence about its

role in growth specifically in crustaceans so a more detailed study of CypA is needed to confirm the correlations suggested by this study.

5.5.3.7 Acyl CoA desaturase (SCD-1)

Research findings have also suggested that MSTN has been involved in the fat regulation as any significant loss of MSTN seems to entail a reduction in fat accumulation through a decrease in the mRNA transcript of fat-regulating genes including SCD-1 (Dobrzyn et al., 2004; Ntambi & Miyazaki, 2003) and leptin (McPherron & Lee, 2002). The down regulation of SCD-1 in bovines for example, leads to disruption of fatty acid synthesis as the gene related to synthesis is also down-regulated while triggering the oxidation-related genes (Kadegowda, Burns, Pratt, & Duckett, 2013). Similarly, the mice identified with a natural mutation in SCD-1 and also with the silenced SCD-1 (transgenic mice) show the down-regulation of lipid synthesis and up-regulation of lipid oxidation related genes (Ntambi et al., 2002). This portrays the ultimate role of SCD-1 as a control point in the lipogenesis process (Ntambi, 1999). Moreover, enzyme regulating lipolysis and fatty acid oxidation has also been shown to increase when there is a reduction in MSTN of mice (Zhang et al., 2012). These previous findings in vertebrates could support the results of our current study. Wherein, MSTN was shown to be closely associated with fat regulation with the down-regulation of *Mr*MSTN leading to a reduction in SCD-1 expression. However, further studies can be performed to elaborate research of the MSTN role in fat regulation in other prawns.

5.6 CONCLUSION

Overall, we have successfully elucidated the specific role of *Mr*MSTN through dsRNA silencing and subsequent histology analysis. The onset of muscle regeneration subsequent to the down-regulation of *Mr*MSTN strongly supports that it serve as a negative growth regulator as in the vertebrates. In addition, this study has also provided solid evidence of how a cluster of growth-regulating genes respond particularly when a negative growth regulator has been silenced. These findings could help in the development of a growth booster to trigger and suppress a set of growth regulating genes so as to favour longer-term muscle growth and development. Moreover, the similar high expression of tropomyosin and arginine kinase on 14 d.p.i is something to ponder in future as both of these are allergen contributors as well as carry important roles in growth and immunity respectively. In addition, the insights into the co-regulation of MSTN and fat metabolism genes are also advantageous in developing an ideally healthy prawn with high growth and low fat content in future. In summary, this study paves a route to further research in promoting *M. rosenbergii* growth for mass production specifically at the hatchery level.

CHAPTER 6
CONCLUSION

Macrobrachium rosenbergii (*Mr*), commonly known as giant freshwater prawn is a high potential commodity species in aquaculture industry. However, this industry has encountered constraint including restriction in growth especially at the hatchery level breeding and susceptibility to diseases that lead to growth deficiencies. Thus, there is an urge to increase the productivity of the growth despite the outbreak of diseases and the growth restrictions. In achieving this ultimate objective, we have postulated that myostatin (MSTN) can be a growth potential gene which can be exploited further to promote growth as well as increase the productivity, therefore it has been chosen as the gene of interest of the study. MSTN is a growth regulating gene belonging to the TGF-β superfamily and carries a unique role in which it serves as a negative growth regulator. The gene has been well established in vertebrates but remain to-be well defined in invertebrates.

The current study was initiated to characterize MSTN gene and to elucidate its intense functional role in *M. rosenbergii* (*Mr*MSTN). Besides that, this study has also attempted to have initial clarity on its influence on other growth regulating genes. The preliminary role of *Mr*MSTN has been reported based on the mRNA regulation patterns on various aspects which include different tissues, at different life stages and in the presence of growth affecting disease. Further down, dsRNA silencing was performed to define the extensive role of *Mr*MSTN in which it was supported by the subsequent gene expression study and the histological analysis. Moreover, the study has been carried ahead by profiling the regulation pattern of mRNA transcripts of a cluster of growth regulating genes in the dsRNA-*Mr*MSTN silenced samples. This aids in elucidating the secondary role of *Mr*MSTN besides proposing a possible co-relation among the selected growth

regulating genes which comes from varies functional group including growth, immunity, reproduction and fat metabolism.

Looking into more specifically, we have successfully achieved the first objective of the study that is to identify and characterize *Mr*MSTN gene for *M. rosenbergii*. The isolated full length *Mr*MSTN consist of 1619 bp with an open reading frame (ORF) of 945 bp that encodes 315 amino acids. It encompasses 5' UTR region, 3' UTR region, N-terminal propeptide domain and C-terminal mature peptide domain. The isolated mature peptide, which actively involved in the mRNA transcription consisted of all the conserved TGF-β superfamily characteristic including cysteine residues and the RXXR proteolytic cleavage region where the 'XX' denotes asparagine (N) and arginine (R). The multiple alignment and phylogenetic tree construction shows that *Mr*MSTN is closely related to the crustacean MSTN group and also shows correlation with vertebrates MSTN and the other TGF-β family member, GDF 11.

In the second objective of the study, we have profiled mRNA transcription regulation patterns in different tissues, at different life stages as well as challenged *M. rosenbergii* upon infection with Infectious hypodermal and hematopoietic necrosis virus (IHHNV). The fundamental tissue distribution analysis shows that *Mr*MSTN was ubiquitously expressed in all tissues tested with the highest mRNA expression in muscle followed by heart, intestine and haemocyte. Meanwhile the *Mr*MSTN regulation at different life stages shows subsequent up and down regulation patterns in progressive life stages. The expression profiling pattern obtained was in accordance with the muscle development and physical activity. Following that, upon IHHNV challenge the *Mr*MSTN expression was also significantly altered during the time interval of the study.

The overall gene expression studies have suggested the preliminary role of *Mr*MSTN to serve as a negative growth regulator. This supports the previously reported MSTN gene expression studies in other crustaceans which solely focus on the different molting stages. Moreover, this study has also provided an initial correlation between the growth affecting infection and the growth regulating gene. The significant changes of *Mr*MSTN expression could favour muscle mass reduction since prolonged IHHNV infection leads to runt deformity syndrome. However, an extensive study is vital to further support this hypothesis.

Following that, we have performed the *in vivo* dsRNA mediated *Mr*MSTN gene silencing to define the *Mr*MSTN role more robustly which constructs the third objective of the study. Further downstream analysis including the gene expression and the histological analysis provides support towards elucidating the extensive role of *Mr*MSTN. The *Mr*MSTN was successfully silenced in which the expression was significantly down-regulated compared to the controls. Besides that, the first documented histological analysis of the dsRNA-*Mr*MSTN challenged muscle tissue evidenced the presence of abundant nuclei cells and the generation of new fibers.

The current dsRNA gene silencing of *Mr*MSTN has supported the occurrence of muscle regeneration as similar to the role of MSTN reported in vertebrate system. Hence, the role of *Mr*MSTN was confidently defined to serve as a growth inhibiting gene in *M. rosenbergii* and could provide insight of its role in invertebrates especially crustaceans. Besides that, the findings also support the preliminary role proposed based on the gene regulation studies in Chapter 2. However, further similar study in other crustaceans are needed to validate the general role of MSTN in invertebrates specifically in crustaceans.

With the ultimate role of *Mr*MSTN being characterized, we further looked into the downstream effect of dsRNA-*Mr*MSTN silencing towards other growth regulating genes. This study has attempted to find the correlation between *Mr*MSTN and other growth regulating genes alongside illuminating the possible role in other mechanisms which include fat regulation, reproduction and immune regulation. The correlation study was conducted with seven genes representing four functional groups that are growth regulation, fat regulation, reproduction and immunity. Overall, as the *Mr*MSTN was down-regulated, the regulation of all the seven genes were altered. The changes in the regulation may perhaps of the direct influence from the silencing effect or the subsequent muscle regeneration that took place. Thus, this preliminary data provides a fundamental pace for further research to elucidate the secondary role of *Mr*MSTN in near future.

In conclusion, the MSTN gene has been successfully identified, characterized and its comprehensive role has been elucidated alongside reporting its downstream effects on a cluster of growth regulating genes in this study. The current findings will be very useful in further expanding the research at the hatchery level specifically transforming the dsRNA silencing study on large-scale of prawn culture. This could aids in achieving the ultimate target that is to promote growth and development of *M. rosenbergii* and also to increase the production in the long term. The successful outcome will be very promising towards increasing the production and thus will be very beneficial to the farmers and also to the country. Moreover, extensive research can be extended to synthesize biomarkers especially in detecting the growth affecting pathogenic infection as it could show immediate alteration in the MSTN regulation. Thus, MSTN is a potential growth regulating gene that can be further explored especially using "dsRNA gene silencing" as a promising tool, towards achieving ideal 'double-muscle' mass *M. rosenbergii* in a massive scale in the near future.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications:

Sarasvathi Easwvaran, Subha Bhassu, Mary Beth Baccano Maningas, Rofina Yasmin Othman. Myostatin, a potential growth regulating gene in Giant River Prawn, *Macrobrachium rosenbergii*. Journal of World Aquaculture Society, 46 (6):624-34.

Sarasvathi Easwvaran, Subha Bhassu, Mary Beth Baccano Maningas, Rofina Yasmin Othman. Myostatin, a prospective target for gene silencing for growth development in *Macrobrachium rosenbergii* and its influence in the growth regulation. (*In preparation*)

Jesu Arockiaraj, Puganeshwaran Vanaraja, **Sarasvathi Easwvaran**, Arun Singh, Tahereh Alinejaid, Rofina Yasmin Othman, Subha Bhassu: *Gene profiling and characterization of arginine kinase-1 (MrAK-1) from freshwater giant prawn (Macrobrachium rosenbergii).* Fish &amp Shellfish Immunology, 31(1):81-9, 07/2011.

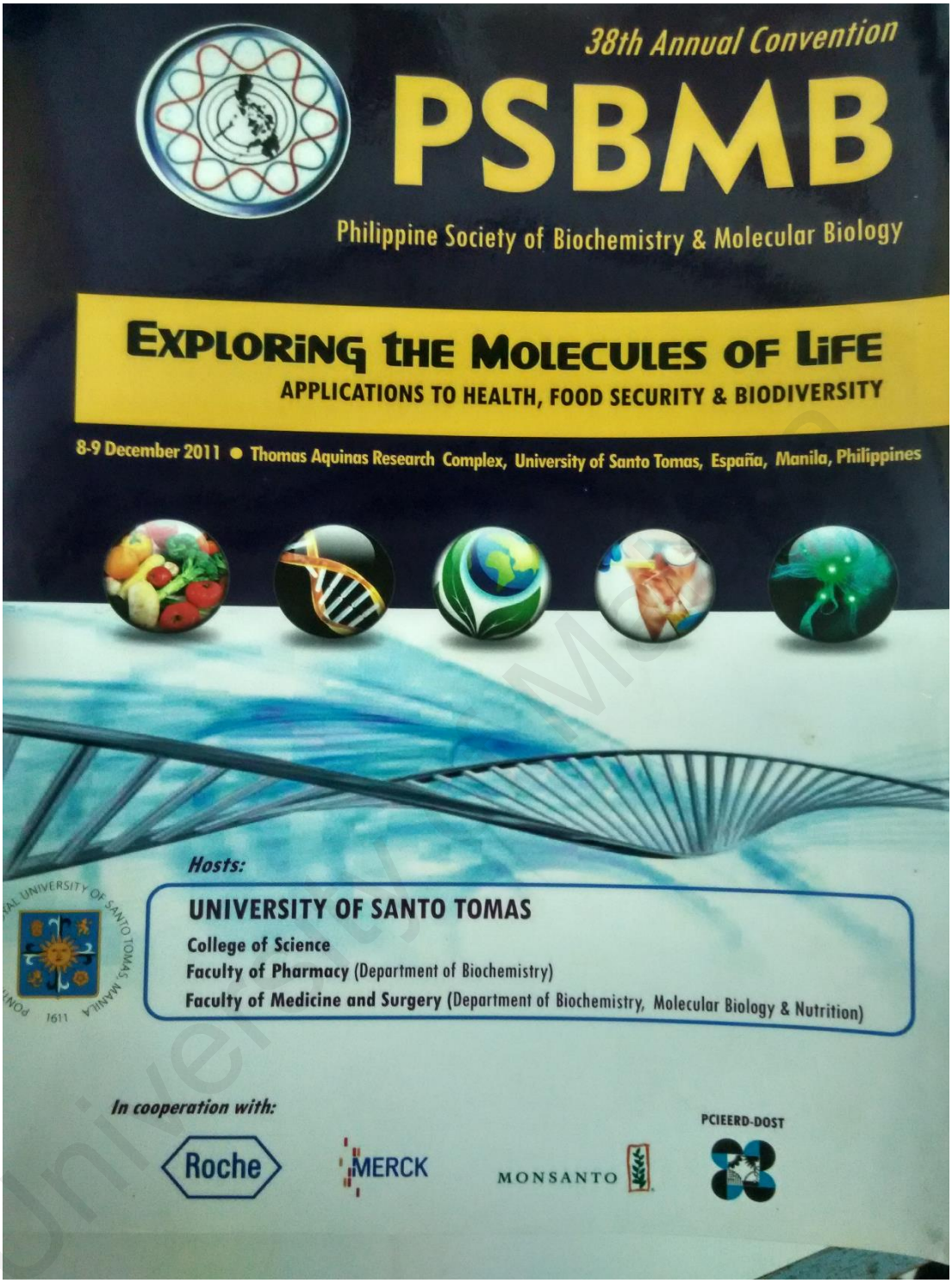
Paper / Poster presentations:

Paper presentation entitled:

"The Discovery of a Novel Myostatin Gene in Giant Freshwater Prawn, *Macrobrachium rosenbergii* Upon IHHNV infection", from 10^{th} – 13^{th} December 2013, at Asian Pacific Aquaculture 2013, Ho Chi Minh City, Vietnam.

Poster presentation entitled:

"Molecular Characterization of Myostatin-like Gene (*Mr*MSTN) from Giant Freshwater Prawn, *Macrobrachium rosenbergii*" from 8^{th}- 9^{th} December 2011 at 38^{th} Annual PSBMB Convention, Thomas Aquinas Research Complex, University of Santo Tomas, Manila, Philippines.


38th Annual Convention
PSBMB
Philippine Society of Biochemistry & Molecular Biology
EXPLORING THE MOLECULES OF LIFE
APPLICATIONS TO HEALTH, FOOD SECURITY & BIODIVERSITY
8-9 December 2011 • Thomas Aquinas Research Complex, University of Santo Tomas, España, Manila, Philippines
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Faculty of Medicine and Surgery (Department of Biochemistry, Molecular Biology & Nutrition)
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Poster Presentations

Food

PF-37 MOLECULAR CHARACTERIZATION OF MYOSTATIN-LIKE GENE (*Mr*MSTN) FROM GIANT FRESHWATER PRAWN, *Macrobrachium rosenbergii*

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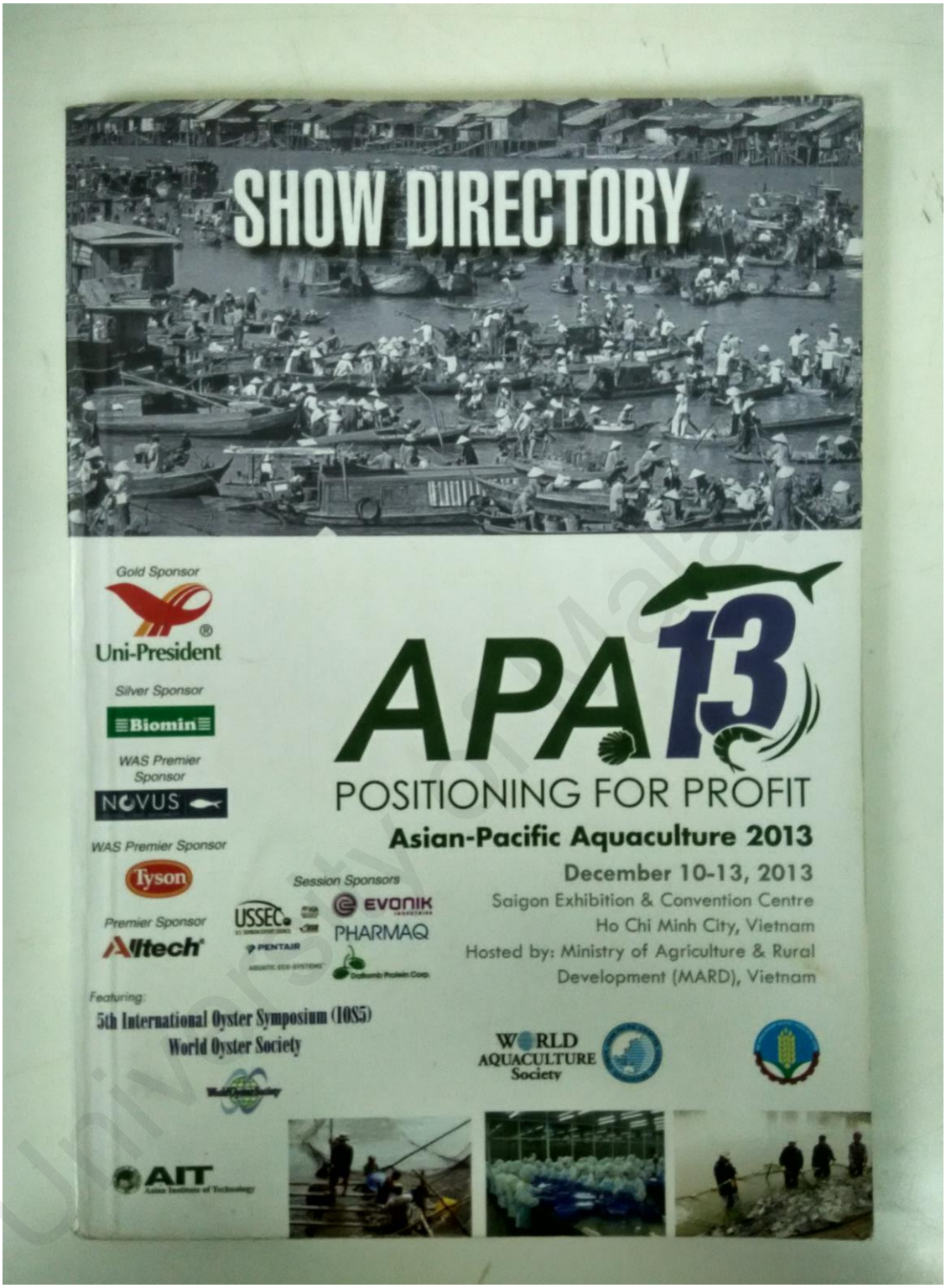
Macrobrachium rosenbergii is the species most favoured for freshwater shrimp farming purposes and remains by far the major subject of cultivation for commercial farming. However, like any other crustacean there is dearth of information in the immune system of this species. Here we cloned and characterized for the first time a cDNA encoding Myostatin (MSTN) – like gene from *M. rosenbergii* (*Mr*MSTN). The *Mr*MSTN like gene consist of 885 base pairs (bp), encoding for 290 amino acids (aa). The current putative *Mr*MSTN consist of an incomplete TGF-β propeptide domain at aa 49-187 and a complete TGF-β mature peptide domain at 200-295 aa sequence. The proteolytic cleavage region, RNRR (Arg^{182}-Gln^{183}-Arg^{184}-Arg^{185}) was identified in the putative *Mr*MSTN. The putative *Mr*MSTN exhibited highest identity/similarity (79% / 85%) with *P. japonica*. The putative 3D structure illustrate a mature protein domain, made up of 6 β-sheets and no trace of α-helix structure. Multiple alignments showed high conservation at the C-terminal region encompassing conserved proteolytic cleavage site (RXXR) for its maturation and nine cysteine residues for disulphide bridges compared with the propeptide domain. Phylogenetic tree construction established very close phylogenetic relationship with 99% homology between putative *Mr*MSTN and *P. japonica* MSTN. All the established crustacean invertebrates MSTN are clustered within a single clade with good bootstrap support of 100%. The molecular cloning and characterization of *Mr*MSTN showed that it isorthologous to other invertebrate myostatin-like protein, higher vertebrate and lower vertebrate groups. Our findings, supports the importance of MSTN in muscle growth and development in invertebrates, as it does in the vertebrate system.



Asian-Pacific Aquaculture 2013 - Meeting Abstract

THE DISCOVERY OF A NOVEL MYOSTATIN GENE IN GIANT FRESHWATER PRAWN, Macrobrachium rosenbergii UPON IHHNV INFECTION

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Macrobrachium rosenbergii is the most favored species and remains by far the major commodity cultivated for freshwater commercial farming. However there is dearth of molecular information in the growth and development of this species. This species also facing low growth upon muscle related infection. Therefore research on key regulatory of growth gene is important in line with improving the growth in prawn. The present study focused on molecular cloning and characterization of myostatin gene in *M. rosenbergii* (*Mr*MSTN) followed by mRNA transcription in different life stages and upon IHHNV infection.

M. rosenbergii of adult stage (15-20g) were obtained live from local hatchery in Malaysia and acclimatize accordingly before proceed to RNA extraction (pooled muscle tissue of 3 adult), cDNA transcription and PCR. Obtained cDNA cloning sequence proceeded to bioinformatics characterization. Gene expression was prior to RNA extraction (pooled tissue of 3 adult). For tissue distribution, different tissues were collected from three adults. As for gene expression of different life stages, pool of larvae (Day 1) was snap frozen into liquid nitrogen, and three individual from Post larvae (Day 60), Juvenile(approximately 5 g) and Adult (10-15g) were collected and acclimatized accordingly. For challenge study, prawns were infected with IHHNV and muscle sample were collected pre (0 hours) and post injection (3, 6, 12, 24 and 48 hours).

Here we have successfully isolated cDNA encoding Myostatin (MSTN) gene from *M. rosenbergii*. The *Mr*MSTN gene consists of 1619 base pairs (bp) with an open reading frame (ORF) of 945 bp that encodes 315 amino acids.The proteolytic cleavage region, RNRR (Arg197-Gln 198-Arg199-Arg200) was identified in the putative *Mr*MSTN. The putative 3D structure illustrates the mature protein domain, made up of 8 β- sheets and single α-helix structure (Fig 1). Multiple alignments showed high conservation at the C-terminal region encompassing conserved proteolytic cleavage site (RXXR) and nine cysteine residues. Phylogenetic tree illustrates *Mr*MSTN clustered in a single blade with other crustacean MSTN where *L. vannamei* and *P. monodon* being closely related species. *Mr*MSTN expression shows ubiquitous expression in all the tissues with largest quantity shown in muscle followed by in intestine, heart and haemocyte (Fig 2). Preliminary report on expression of *Mr*MSTN in different life stages and IHHNV challenged *M.rosenbergii* showed significant ($P < 0.05$) differences along the growth progression and post-hour challenge respectively. Our findings, supports the importance of MSTN in muscle growth and development in invertebrates, as in the vertebrate system.

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Myostatin: A Potential Growth-Regulating Gene in Giant River Prawn, *Macrobrachium rosenbergii*

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Abstract

Growth retardation and diseases hindering growth are constraints faced by *Macrobrachium rosenbergii* and thus urge an in-depth study on the growth and growth-related genes to improve this species. This study focused on molecular cloning and characterization of the myostatin gene in *M. rosenbergii* (*Mr*MSTN) followed by mRNA transcription in different life stages and upon infectious hypodermal and hematopoietic necrosis virus (IHHNV) infection. We have characterized a full-length *Mr*MSTN gene encoding 1619 base pairs (bp) with an open reading frame of 945 bp that encodes for 315 amino acid residues. *Mr*MSTN retains all the conserved characteristics belonging to TGF-β superfamily including propeptide and mature peptide domain, cysteine residues and a proteolytic cleavage site, RXXR where "XX" denotes Asparagine and Arginine. *Mr*MSTN gene is ubiquitously expressed in all the tissues of healthy adults, with the highest expression observed in muscle. Moreover, the *Mr*MSTN transcripts showed significant ($P < 0.05$) changes in different life stages of *M. rosenbergii* and IHHNV-challenged prawns. The results indicate the possible functional role of *Mr*MSTN as a negative growth regulator. Thus, *Mr*MSTN can be exploited further to enhance the growth and can be used as a biomarker to address the growth deficiency-related problems in *M. rosenbergii*.

Macrobrachium rosenbergii is commonly known as "giant river prawn," a commercially important cultivable food species of South and Southeast Asia. Prawn farming has increased in various countries (FAO 2012). Mather (2008) has reported that extensive research has been carried out on this species after its breeding and hatchery techniques were successful. However, the shrimp industry faces constraints such as low growth and susceptibility to diseases (Valderrama and Anderson 2012) that have significant impacts on prawn growth. The growth in prawns depends on their efficiency in muscle restoration after each molting process. Therefore, research into key regulatory genes that are responsible for muscle restoration is of obvious importance.

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Myostatin (MSTN) is one of the important genes closely related to growth in muscles alongside others such as crustacean hyperglycaemic hormones, actin, tropomyosin, ecdysteroids, fatty acid elongase, crustacean neuropeptides, and molting inhibiting hormone (De-Santis and Jerry 2007).

Macrobrachium rosenbergii was found to be susceptible to bacterial and viral infections including infectious hypodermal and hematopoietic necrosis virus (IHHNV) infection (Hsieh et al. 2006; Nita et al. 2012). IHHNV has been reported to infect many penaeid species such as *Litopenaeus stylirostris, Litopenaeus vannamei*, and *Penaeus monodon* and induce slow growth (Bell and Lightner 1984; Kalagayan et al. 1991; Lightner et al. 1992; Hsieh et al. 2006; Rai et al. 2008). IHHNV is a single-stranded DNA virus belonging to the *Parvoviridae* family and has been reported to cause 90% mortality at the juvenile stage (Lightner 1996; Briggs et al. 2005). Runt-deformity syndrome has been observed in IHHNV-infected individuals; this syndrome leads to suffocation and growth reduction (Lightner 1996). The postlarval and subadult stages of *M. rosenbergii* have been observed to be more vulnerable to IHHNV infection, which causes growth deformities in the fourth to sixth abdominal segments and tail fan (Hsieh et al. 2006). Therefore it is essential to gain a better knowledge on the correlation between the growth-associated genes such as MSTN and IHHNV infections in *M. rosenbergii.*

MSTN has been identified as a growth differentiation factor-8 (GDF-8) and known to function as a negative growth regulator. According to Acosta et al. (2005), it inhibits or restricts the growth and development of muscle in vertebrates. Transforming growth factor β (TGF-β) contains MSTN abundantly in an inactive form. Upon proteolytic cleavage at the conserved RXXR, a proteolytic recognition site by propeptide convertase, it produces an active mature peptide of MSTN (Kim et al. 2010). The mature peptide contains all the conserved cysteine residues that are involved in disulfide bonding, a characteristic feature of MSTN-like and inhibin genes (Kim et al. 2010). Disruption in the function of MSTN either by natural mutation or MSTN gene knockout promotes an increase in muscle mass by either hyperplasia or hypertrophy or both. Earlier researchers (Grobet et al. 1997; Mcpherron et al. 1997) demonstrated that silencing MSTN increases the muscle mass in mice and cattle, respectively.

MSTN has been extensively studied in vertebrates as a negative regulator, but the information is limited in invertebrates. Hence, it is necessary to elucidate its potential role in prawns. Knowing about MSTN distribution in tissues, various life stages of *M. rosenbergii*, and its regulation upon infection will provide insights into its importance in prawn developmental stages and upon growth-affecting infection. Keeping this in mind, this study was carried out to demonstrate the gene expression profile of MSTN from healthy, IHHNV-infected, and various developmental stages of *M. rosenbergii*. This study provides basic information of the MSTN mRNA profiling for *M. rosenbergii* alongside establishing the preliminary report of its influence in growth progression and correlation with a growth-affecting infection. This will be very useful in developing the gene as a biomarker and to enhance growth that could influence the turnover of product in the aquaculture industry.

Materials and Methods

Primer Designing

Three different unigenes of MSTN partial sequences were obtained from *M. rosenbergii* transcriptome database GenBank (Mohd-Shamsudin et al. 2013), which had been generated utilizing Illumina's Solexa sequencing technology. The unigenes were then aligned using a MSTN-like gene from the Morotage shrimp, *Pandalopsis japonica*, as a reference gene (GenBank Accession No. GU130188) in MEGA 4.0 software (Table 1). Six primer pairs were designed using Primer3Blast software to sequence the full length of the *Mr*MSTN gene. The random amplification of cDNA end (RACE) and real-time primers were designed using IDT SCI TOOLS programs (http://www.idtdna.com/SciTools/SciTools.aspx) (Table 2).

TABLE 1. *Unigenes arranged in position based on multiple alignments with reference gene* Pandalopsis japonica.

No.	Unigene	Query position	*P. japonica* (subject position)
1	Unigene16954_All	174–351	43–220
2	Unigene9180_All	1–436	218–656
3	Unigene6326_All	1–302	688–989
4	Unigene74700_All	3–158	835–989

TABLE 2. *Primer set designed for sequencing of MSTN gene primer for RT-PCR, primers for RACE, and primer for real-time.*

	Sequence	T_A (°)
RT-PCR		
MSTN A1 F	TTAACTCCACCCGATATGACAGGGG	57.0
MSTN A1 R	GCCGGTTCATCGTCATTATATGGGG	
MSTN B1 F	CGCCAGCGGGGCTCAGAATT	49.8
MSTN B1 R	GGTTGCGCCCTCGACGACTT	
MSTN C1a F	TGCTGTCGTTACCCTCTCTCGG	49.6
MSTN C1a R	CAGGGACCGTGTTTGGCGCT	
MSTN C3b F	ATGAACAGCAGCAGCGCCAAA	52.2
MSTN C3b R	GAGCACCCACAACGATCCACG	
MSTN C5 F	TAGTAGCACCCAAGGTCTACGA	55.3
MSTN C5 R	TAAGAGCACCCACAACGATCCA	
MSTN D1 F	CACACGGAGGAAAGTCGTCGAG	60.6
MSTN D1 R	ACCGAGAGAGGGTAACGACAGC	
RACE		
5′ RACE R	GCCGGTTCATCGTCATTATATGGGG	57.0
3′ RACE F	TAGTAGCACCCAAGGTCTACGA	55.3
Real-time		
*Mr*MSTN F	TAGTAGCACCCAAGGTCTACGA	60.0
*Mr*MSTN R	TAAGAGCACCCACAACGATCCA	60.0
EF F	ACTGCGCTGTGTTGATTGTAGCTG	60.0
EF R	ACAACAGTACGTGTTCACGGGTCT	60.0

RACE, random amplification of cDNA end; RT-PCR, real-time polymerase chain reaction.

Sample Collection

Shrimp for cDNA Cloning and Tissue Distribution. Macrobrachium rosenbergii adults (15–20 g) were obtained in live condition from a local hatchery at Negeri Sembilan, Malaysia. The prawns were transported to the laboratory and reared in flat-bottomed glass tanks (300 L) with aerated and filtered freshwater for a week of acclimatization. During acclimatization, the prawns were fed once a day with commercial prawn pellets at a satiation level. The water was changed every day, and the quality parameters were maintained at the prescribed level (Buikema et al. 1982). The prawns were treated with care following all the ethical procedures of the university. The prawns were sacrificed within 10 sec by dissecting the head. Pooled muscle tissues from three *M. rosenbergii* adults were used for cloning of the *Mr*MSTN gene. Tissues including hemocytes, eyes, hearts, stomachs, hepatopancreas, gills, intestines, pleopods, and muscles pooled from three adults were used for the *Mr*MSTN gene expression profile in *M. rosenbergii.*

Different Life Stages of M. rosenbergii. *Macrobrachium rosenbergii* at different life stages were collected from a freshwater prawn breeding hatchery at Negeri Sembilan, Malaysia. A pool of larvae (day 1) were collected from the hatchery and immediately frozen in liquid nitrogen and then stored at −80 C for further RNA extraction. Postlarvae (day 60), juveniles (ca. 5 g), and adults (ca. 15 g) were collected and acclimatized in the laboratory for 7 d in flat-bottomed glass tanks (300 L) with aerated and filtered freshwater. Muscle tissue from three individuals for each stage was pooled for further RNA extraction followed by first-strand cDNA synthesis. Three different cDNA templates were then used for further quantitative polymerase chain reaction (PCR) analysis. The obtained data were expressed as relative fold of one sample (lowest expression) as mean ± SD.

Macrobrachium rosenbergii *and IHHNV Challenge.* To study the IHHNV-induced mRNA expression analysis, the prawns were injected with IHHNV. Briefly, IHHNV-infected prawn tail tissue, tested positive by nested PCR, was homogenized in sterile 2% NaCl (1:10, w/v) solution and centrifuged at 2991 *g* for 5 min at 4 C. The supernatant was filtered through a 0.45-μm filter and used for injecting (100 μL/10 g prawn) the prawns. Tissues (hemocytes, eyes, hearts, stomachs, hepatopancreas, gills, intestines, pleopods, and muscle) were collected before (0 h) and after injection (3, 6, 12, 24, and 48 h) and were immediately snap-frozen in liquid nitrogen and stored at −80 C until the total RNA was isolated. Using a sterilized syringe, the hemolymph (0.2–0.5 mL per prawn)

was collected from the prawn heart and immediately centrifuged at 3000 *g* for 10 min at 4 C to allow hemocyte collection for total RNA extraction. The collected samples at each time point were qualitatively tested for positive IHHNV infection using World Animal Health Information System forward primer (5′-CGGAACAC AACCCGACTTTA-3′) and reverse primer (5′-GGCCAAGACCAAAATACGAA-3′), which amplify a 389-base pair (bp) fragment (OIE 2003, 2009).

RNA Purification and cDNA Synthesis

Total RNA was isolated from *M. rosenbergii* tissues using TRIZOL reagent following the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). Total RNA was treated with RNase-free DNA set (5 Prime GmbH, Hamburg, Germany) to remove the contaminating DNA. The total RNA concentration was measured spectrophotometrically (NanoVue Plus Spectrophotometer, GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK). First-strand cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) followed by oligo-dT (20) VN primer in accordance with the manufacturer's protocol.

Cloning of Mr*MSTN cDNA*

PCR amplification was carried out in the following conditions: 1.2 µL $MgCl_2$, 3.0 µL 10× Buffer, 1.0 µL dNTPs, 0.3 µL forward and reverse primers, and 0.25 µL Go Taq Polymerase in a total reaction volume of 10 µL. The PCR profile was as follows: initial denaturation at 94 C for 5 min, 35 cycles at 94 C for 30 sec, annealing temperature (Table 2) for 30 sec, and 72 C for 30 sec, final extension for 7 min at 72 C.

PCR products were separated with 1.5% agarose gel electrophoresis and identified on the UV transilluminator after staining with ethidium bromide. The PCR products with expected sizes were purified using a Gel Extraction Kit (Geneall, Seoul, Korea), ligated into the TA plasmid vector using the pGEM-T Easy Cloning Kit (Promega) and transformed into a One Shot Top 10 *Escherichia coli* strain (Invitrogen). Nucleotide sequences of the cloned cDNAs were determined using an automated DNA sequencer (ABI Biosystems, Carlsbad, CA, USA).

A 5′ and 3′ RACE was performed to amplify the 5′ and 3′ regions using a 5′ and 3′ RACE Gene Racer Kit (Promega) according to the manufacturer's instructions; RACE primers are shown in Table 2. RACE conditions were as follows: 0.4 µL RACE template cDNA was used in each reaction, with 8 pmol of each gene-specific (Table 2) and kit primer, and other components identical to the initial PCR reactions (see above). After denaturation at 94 C for 3 min, 35 cycles at 94 C for 30 sec, at the annealing temperature for 30 sec (Table 2), and at 72 C for 30 sec were completed. Final extension was for 7 min at 72 C. All the products were cloned and sequenced as described earlier.

Bioinformatics Analysis

The basic local alignment tool (BLAST) program was used to search similar nucleotide and protein sequences to the MSTN (Altschul et al. 1990). The open reading frame (ORF) and amino acid sequence of MSTN were obtained using DNAssit 2.2. Characteristic domains or motifs were identified using the PROSITE profile database (Bairoch et al. 1997).

Quantitative Real-time PCR Analysis of Mr*MSTN mRNA Expression*

Quantitative real-time PCR analysis (qRT-PCR) was carried out using an ABI 7500 Real-time Detection System (Applied Biosystems, Foster City, CA, USA) in 20 µL reaction volume containing 4 µL of cDNA (50 ng) from each tissue, 10 µL of Fast SYBR Green Master Mix, 0.5 µL of each primer (20 pmol/ml), and 5 µL dH_2O. The qRT-PCR cycle profile was 1 cycle at 95 C for 10 sec, followed by 40 cycles at 95C for 15 sec, 60 C for 60 sec, and 72 C for 10 sec. The same qRT-PCR cycle profile was used for the internal control gene, elongation factor-1 (EF-1). The primers used in this study are presented in Table 2. EF-1 primers were designed based on EST similar to EF-1α from *M. rosenbergii* (GenBank Accession No. EL609261). After the PCR program,

the data were analyzed with ABI 7500 SDS software (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. The comparative CT method (2^{-ddCT} method) was used to analyze the expression level of *Mr*MSTN (Livak and Schmittgen 2001). All data were given in terms of relative mRNA expressed by means ± SD. To compare relative *Mr*MSTN mRNA expression, a statistical analysis was performed using a one-way ANOVA, and mean comparisons were performed by Tukey's Multiple Range Test using SPSS 11.5 at the 5% significant level.

Results

Cloning and Characterization of MSTN-like Gene from M. rosenbergii

This study describes an initial attempt of isolation of MSTN from *M. rosenbergii* (*Mr*MSTN). This was achieved using specific primers which are designed based on the unigene templates obtained from the transcriptome database of *M. rosenbergii.* The putative *Mr*MSTN (partial sequence submitted, GenBank Accession No. AFP74567.1) sequence contains 1619 bp with an ORF of 945 bp, which encodes 315 amino acids (Fig. 1). The isolated *Mr*MSTN consists of 354 bp 5′ untranslated region (UTR) and 320 bp 3′ UTR obtained from 5′ and 3′ RACE. *Mr*MSTN polypeptide contains a TGF-β propeptide domain or N-terminal domain between 114 and 198 amino acids (aa) and a mature peptide domain or C-terminal domain between 216 and 311 aa. It contains all the conserved properties and active mature peptide region including nine cysteine residues (Cys^{207}, Cys^{216}, Cys^{217}, Cys^{244}, Cys^{248}, Cys^{275}, Cys^{276}, Cys^{308}, and Cys^{310}) and proteolytic cleavage region RNRR (Arg^{197}-Asp^{198}-Arg^{199}-Arg^{200}), which matches the RXXR consensus site.

The amino acid sequence of putative *Mr*MSTN was used as a query to perform a BLASTP search. The putative *Mr*MSTN exhibited the highest identity (72%) with *P. japonica* followed by *L. vannamei* and *P. monodon* (68%). *Mr*MSTN also showed a high level of identity with other crustacean members such as *Homarus americanus* (64%), *Gecarcinus lateralis* (59%), and *Eriocheir sinensis* (55%). All the percentages given are based on the alignment of 98% query coverage with all the crustaceans mentioned above. The putative *Mr*MSTN also showed similarities with lower and higher groups of vertebrates (42–48%) covering 60% of the query sequence. This includes the lower vertebrates *Tribolium castaneum*, *Danaus plexippus*, and *Salmo salar* and the higher vertebrates *Sus scrofa*, *Capra hircus*, *Bos grunniens*, and *Homo sapiens.* Moreover, putative *Mr*MSTN also shows significant level of identity with other TGF-β family members, such as a GDF-11 (28%) from *Danio rerio* and *Sparus aurata*.

Macrobrachium rosenbergii *Myostatin mRNA Expression in Tissues*

The presence of *Mr*MSTN mRNA transcript in various tissues of healthy adult *M. rosenbergii* was quantified using qRT-PCR (Fig. 2). The result shows that *Mr*MSTN was ubiquitously expressed in all the tissues tested. The highest expression was observed in the muscle, followed by the intestine, heart, and hemocyte. Meanwhile, lower expression of *Mr*MSTN was detected in the stomach, gills, pleopods, and hepatopancreas, whereas eyes show the lowest expression. A statistical analysis showed that *Mr*MSTN mRNA expression was significantly higher ($P < 0.05$) in the muscle. Therefore, muscle tissue was selected as a target tissue to further quantify the *Mr*MSTN mRNA transcript at different life stages and postinfection with IHHNV.

Macrobrachium rosenbergii *Myostatin mRNA Expression at Different Life Stages*

In this analysis, the expression of *Mr*MSTN at different life stages, including larvae, postlarvae, juvenile, and adult (Fig. 3) of *M. rosenbergii* was reported. The data showed that the expression of *Mr*MSTN significantly ($P < 0.05$) varies at different stages of growth progression. *Mr*MSTN was highly expressed during the larval stage, followed by a drastic decrease in the postlarval stage. However, the expression increased again three fold at the juvenile stage; and finally at the adult stage, the expression decreased.

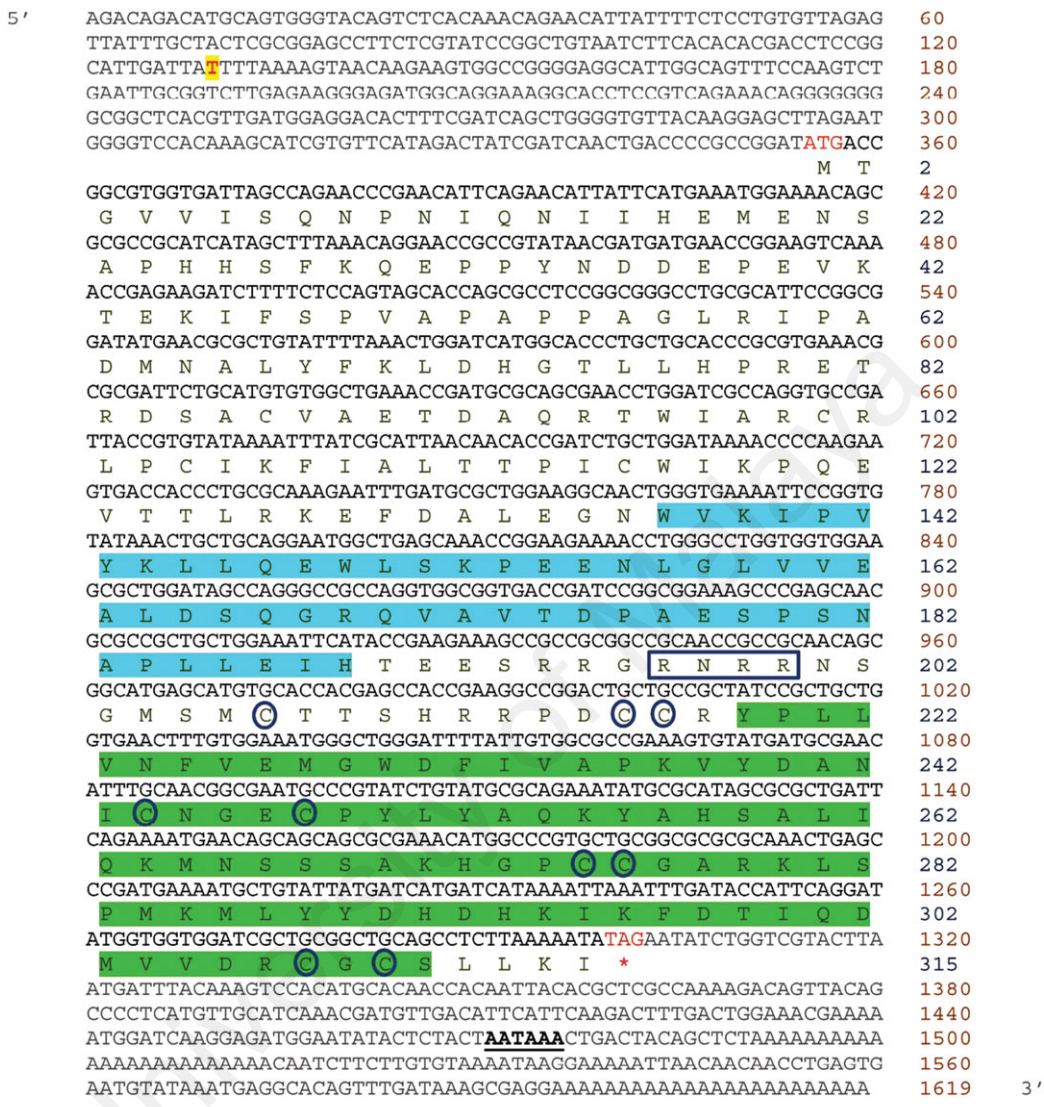

FIGURE 1. *Nucleotide and deduced amino acid sequences of* Mr*MSTN. The brown color numbers indicated on right side of each row represent the position of nucleotide and the black color numbers represent the amino acid position. The furin recognition and cleavage site (RXXR) are indicated in blue color box. Conserved seven cysteine residues are indicated in circle and two additional Cys residues at 207 and 217 also indicated in circle. The TGF-B propeptide region (N-terminal) is highlighted in blue, and the TGF-B mature domain region is highlighted in green. The start and stop codons are highlighted in red. The polyadenylation site is underlined.*

Macrobrachium rosenbergii *Myostatin mRNA Expression in Muscle after IHHNV Challenge*

Because IHHNV is known to affect growth, this study was conducted to analyze the *Mr*MSTN expression pattern upon IHHNV infection (Fig. 4). The organisms challenged with IHHNV were randomly tested for IHHNV positivity at different time points using primer pair from 389 bp product. The results showed positive signs off IHHNV infection (data not shown). *Mr*MSTN expression levels upon IHHNV infection showed a gradual increase with a sharp increase at 6 h postinjection. The level of expression then decreased at 12 h,

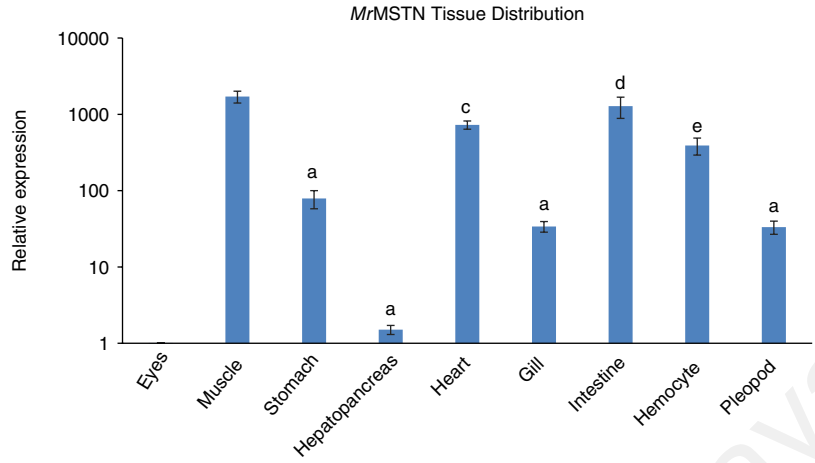

FIGURE 2. *Gene expression patterns of* Mr*MSTN in different tissues of* Macrobrachium rosenbergii *by qRT-PCR.* Mr_*EF (expressing at similar levels in all tissues) served as constitutively expressed control. The statistical analysis was based on comparison of the relative expression ratio of the* Mr*MSTN gene to the* Mr_*EF by calculating* $2^{-\Delta\Delta Ct}$ *values. Data are expressed as a ratio of* Mr*MSTN mRNA expression in eye. The different alphabets are statistically significant at the* $P < 0.05$ *by one-way ANOVA and Tukey's multiple range test.*

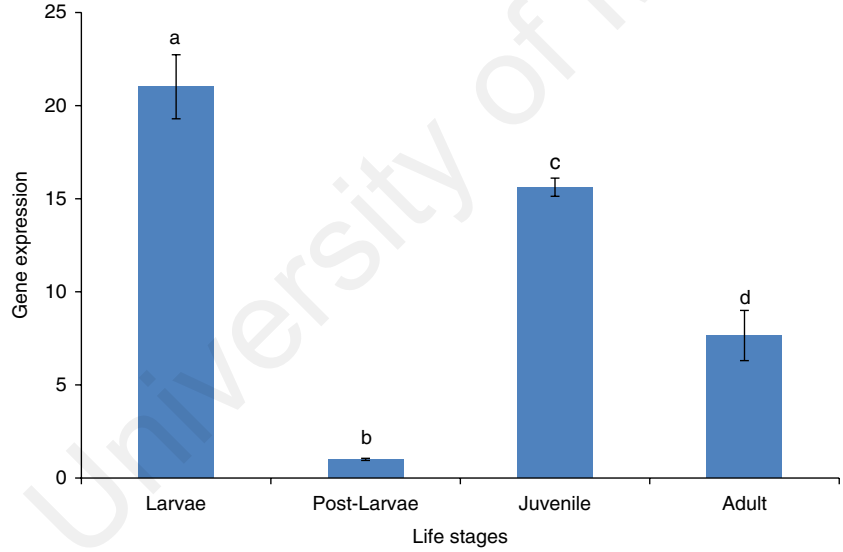

FIGURE 3. *Gene expression patterns of* Mr*MSTN in differential life stages of* Macrobrachium rosenbergii *by qRT-PCR.* Mr_*EF (expressing at similar levels in all tissues) served as constitutively expressed control. The statistical analysis was based on comparison of the relative expression ratio of the* Mr*MSTN gene to the* Mr_*EF by calculating* $2^{-\Delta\Delta Ct}$ *values. Data are expressed as a ratio of* Mr*MSTN mRNA expression in postlarvae. The statistically significant of* Mr*MSTN expression* ($P < 0.05$) *between different life stages are indicated as a, b, c, and d.*

followed by a slower decline at 24 and 48 h. Significant differences ($P < 0.05$) in expression were observed at 3, 6, 12, and 24 h postinjection between the IHHNV-challenged group and the control group.

Discussion

MSTN, a well-known growth-regulating gene, serves as a negative regulator, as it restricts muscle proliferation and development in vertebrate systems (Patel and Amthor 2005). However, its

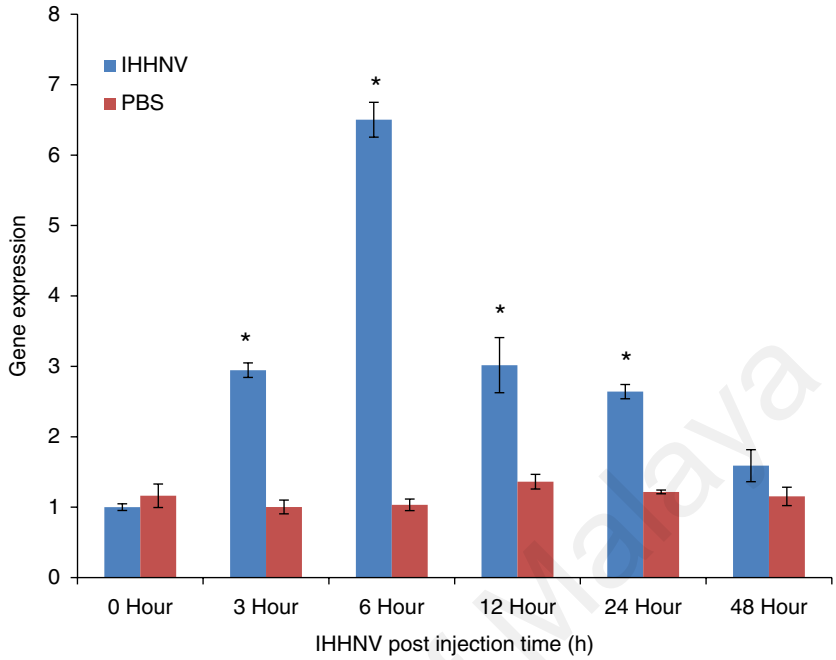

FIGURE 4. *Gene expression patterns of* Mr*MSTN injected with IHHNV and PBS (control) in* Macrobrachium rosenbergii *by qRT-PCR.* Mr*_EF (expressing at similar levels in all tissues) served as constitutively expressed control. The statistical analysis was based on comparison of the relative expression ratio of* Mr*MSTN gene to the* Mr*_EF by calculating* $2^{-\Delta\Delta Ct}$ *values. Data are expressed as a ratio of* Mr*MSTN mRNA expression at 0 h. The statistically significant of* Mr*MSTN expression* ($P < 0.05$) *between the challenged and the control group are indicated with asterisks.*

role in invertebrates has remained much less clear, and more in-depth studies are required to elucidate this. Recently, MSTN has been characterized in invertebrates including *P. monodon* (Santis et al. 2011), *P. japonica* (Kim et al. 2010), *L. vannamei* (Qian et al. 2013), *D. melanogaster* (Lo and Frasch 1999), *A. irradians* (Kim et al. 2004), *E. sinensis* (Kim et al. 2009), and *G. lateralis* (Covi et al. 2008). The current isolated *Mr*MSTN conserved characteristic features including proteolytic cleavage site and cysteine residues. This could suggest that the functional role of MSTN in invertebrates could be similar to vertebrates.

To date, mRNA tissue distribution of MSTN in *P. japonica, P. monodon, H. americanus, L. vannamei*, and *G. lateralis* has been reported in crustaceans. The ubiquitous expression of *Mr*MSTN in both the muscle and nonmuscle tissues are concurrent with the MSTN expression reported for *P. japonica* and *P. monodon* (Kim et al. 2010; Santis et al. 2011). Moreover, the ubiquitous expression of MSTN is also reported for vertebrate systems and other TGF-β family members including *S. salar* (Ostbye et al. 2001) and *D. rerio* (Biga et al. 2005). The presence of MSTN ubiquitous expression suggests its multiple functionality role in both vertebrates and invertebrates. Therefore, further study is required to elucidate the possible functional roles of the MSTN gene in the invertebrate system.

So far, the functional role of MSTN in crustaceans has been reported based on the mRNA transcription at different molting stages, because crustaceans are known to have discontinuous growth and undergo molting (Covi et al. 2010; Kim et al. 2010; Santis et al. 2011; Qian et al. 2013). More specifically, MSTN in crustaceans has been thought to regulate protein turnover during the molting process (Maclea et al. 2010), to regulate expression in muscle tissue (Covi et al. 2008), and to serve as a negative regulator by suppressing MSTN protein turnover in claw

muscle (Covi et al. 2010). On the other hand, MSTN of *P. monodon* (Santis et al. 2011) and *L. vannamei* (Qian et al. 2013) have conversely been reported to aid in muscle growth. Such an inconsistency in the invertebrate MSTN called for more precise elucidation of its role. Thus, this study has elucidated the role of *Mr*MSTN through mRNA regulation in various life stages and upon growth-affecting IHHNV infection.

During the larval stage, the development of *M. rosenbergii* is known to focus on physical changes, as it undergoes approximately 11 zoetal stages (Zoea I–XI), which take 20–35 d before developing into postlarvae (Ling 1969; Nandlal and Pickering 2005). On the other hand, extensive muscles development is observed at the postlarval stage to juvenile stages. Consistent with this, *Mr*MSTN expression was observed to be high in the larval stage but then to decrease drastically in the postlarval stage. Prawns at the adult stage are mainly focused on reproduction as it is documented that female adults have a high tendency to favor reproductive activities over growth development (Ling 1969). Therefore, a slight decline in *Mr*MSTN was observed at the adult stage compared with the juvenile stage. As a whole, the *Mr*MSTN regulation pattern coincides with the growth development in each life stage, suggesting it serves as a negative growth regulator.

This is further supported by the significant sharp increase in expression observed at 6 h IHHNV-postinjection, which also suggests that *Mr*MSTN plays a role in decreasing muscle mass upon the infection. Previous findings have reported an elevated MSTN expression in acquired immune deficiency syndrome–infected humans (Gonzalez-Cadavid et al. 1998) and limb-loosed mice (Wehling et al. 2000), which undergo muscle mass reduction. Thus, this supports current elevated *Mr*MSTN mRNA transcripts and suggests that it could aid in the growth reduction upon prolong infection with IHHNV. Moreover, this will be the first to report a correlation between IHHNV infection and growth at the molecular level.

In conclusion, this study reported a successful cloning and characterization of *Mr*MSTN from *M. rosenbergii*. *Mr*MSTN was found to have the characteristic features of the TGF-β superfamily, including RXXR, proteolytic cleavage sites, and nine cysteine residues. The regulation of *Mr*MSTN transcription at different life stages and upon IHHNV infection strongly suggests that *Mr*MSTN serves as a negative growth regulating gene similar to vertebrates, therefore it influences the growth, development, and muscle proliferation. In addition, the corresponding *Mr*MSTN regulation upon IHHNV infection suggests that it could influence growth reduction in the long term. Thus, *Mr*MSTN can be developed as a biomarker and potential gene manipulation target to aid the aquaculturist in spotting infected prawns and to improve growth. However, further research is needed on gene knockout, physiological changes, and myofibrillar protein contents accompanying current regulation of *Mr*MSTN to determine its role more precisely.

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Gene profiling and characterization of arginine kinase-1 (MrAK-1) from freshwater giant prawn (Macrobrachium rosenbergii)

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abstract

Arginine kinase-1 (MrAK-1) was sequenced from the freshwater prawn Macrobrachium rosenbergii using Illumina Solexa Genome Analyzer Technique. MrAK-1 consisted of 1068 bp nucleotide encoded 355 poly-peptide with an estimated molecular mass of 40 kDa. MrAK-1 sequence contains a potential ATP:guanido phosphotransferases active domain site. The deduced amino acid sequence of MrAK-1 was compared with other 7 homologous arginine kinase (AK) and showed the highest identity (96%) with AK-1 from cherry shrimp Neocaridina denticulate. The qRT-PCR analysis revealed a broad expression of MrAK-1 with the highest expression in the muscle and the lowest in the eyestalk. The expression of MrAK-1 after challenge with the infectious hypodermal and hematopoietic necrosis virus (IHHNV) was tested in muscle. In addition, MrAK-1 was expressed in Escherichia coli by prokaryotic expression plasmid pMAL-c2x. The optimum temperature (30 °C) and pH (8.5) was determined for the enzyme activity assay. MrAK-1 showed significant ($P < 0.05$) activity towards 10e50 mM ATP concentration. The enzyme activity was inhibited by a-ketoglutarate, glucose and ATP at the concentration of 10, 50 and 100 mM respectively. Conclusively, the findings of this study indicated that MrAK-1 might play an important role in the coupling of energy production and utilization and the immune response in shrimps.

1. Introduction

Arginine kinase (AK) plays a central role in both temporal and spatial ATP buffering in cells with high, fluctuating energy requirements (muscle, nerves, etc.). AK catalyzes the reversible phosphorylation of arginine by MgATP to form phosphoarginine and MgADP, thereby regenerating ATP during bursts of cellular activity [1]. AK is the most widely distributed among invertebrates. AK activity has been identified even in protozoa and its gene has been found in the genomes of Paramecium and Tetrahymena, indicating AK has an ancient origin. As a member of the phospha-gen kinase family, it is mainly distributed in invertebrates, and plays a crucial role in the energy mechanism, which is analogous to the creatine kinase (CK) reaction in vertebrates [2]. AK is an ideal paradigm for the classical enzymology of bimolecular reactions [3].

According to Iwanami et al. [4] AK evolved independently at least three times. Firstly, at an early stage of phosphagen kinase evolution (typical AK), second from the cytoplasmic creatine kinase (CK) gene later in metazoan evolution (Stichopus AK) and third from the mitochondrial CK gene in the course of annelid radiation (Sabel-lastarte AK). Iwanami et al. [4] also reported that three AKs (AK1, AK2 and AK3) from cherry shrimp Neocaridina denticulate. Among the three expressed AKs, AK2 is the typical AK, containing the conserved key residues established in Limulus AK for the substrate binding site and expressed among arthropods including crusta-ceans. AK1 and AK3 lacked some of these key residues, indicating a similar evolution to Stichopus AK and Sabellastarte AK. The size of amino acid of these three AKs ranged between 355 and 356 residues and all these AKs showed monomeric nature similar to typical AKs. Moreover, phylogenetic analysis of phosphagen kinases indicated that N. denticulate AK1 and AK3 diverged at the deepest branching point close to the root of the tree and formed a distinct cluster outside the typical AK cluster, which included N. denticulate AK2. Kinetic constants of N. denticulate AKs were similar to those of other AKs. However, activation energy for the transition state of AK1 and AK3 was about 1.5 fold larger than that of AK2.

Most of the AKs are monomers of 40 kDa, while the other phos-phagen kinase, creatine kinase, glycocyamine kinase and lombricine

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kinase are dimeric, or octameric in the case of mitochondrial CK [5,6]. In 1997 an unusual 80 kDa AK with a two-domain structure has been isolated from the primitive sea anemone Anthopleura sp by Suzuki et al. [7]. Interestingly, AK appears to have evolved at least twice during evolution of PKs: firstly at an early stage of phosphagen kinase evolution and secondly, from CK at later time in metazoan evolution [8]. Recently, a few researchers have also discovered AK with 2 domain structure from deep-sea clam Calyptogena kaikoi [9], clams
Solen strictus and Corbicula japonica [10] have an molecular mass of 80 kDa AK. But their enzymatic properties were comparable to those of usual 40 kDa AKs.

Recently, a number of cDNA from cucumber Stichopus japoni-cus [1,3], oyster Crassostrea sp [5], kuruma prawn Marsupenaeus japonicus [6], Callinectes sapidus [11], deep-sea clam Calyptogena kaikoi [9] clams Solen strictus and Corbicula japonica [10] chiton Liolophura japonica and turbanshell Battilus cornutus [12], Cellana grata, Aplysia kurodai [13], horseshoe crab Limulus polyphemus
[14], sea Chinese shrimp Fenneropenaeus chinensis [15], greasy-back shrimp Metapenaeus ensis [16], Litopenaeus vannamei [17] and sea anemone Anthopleura japonicus [18] encoding AKs in major groups of invertebrate have been made available to investigate AK's structure, catalysis mechanism and evolutionary relationship.

A crystal structure for the transition state analog complex of the monomeric AK from horseshoe crab Limulus polyphemus has appeared which suggests that AKs with 2 domains have a unique substrate binding system [19]. These two arginine kinase forms show very similar kinetic properties, such as both require a divalent cation ($Mg^{2þ}$ or $Mn^{2þ}$) for activity. High concentrations of free $Mg^{2þ}$ will inhibit both enzymes. Although no catalytic basis for a functional difference between the two forms was found, it is possible that in vitro differences in stability may reflect stabilities in vivo and thus provide a basis for regulating arginine kinase levels in various tissues thereby giving a selective advantage to species having both forms.

In recent studies, it was demonstrated that the expression of AK correlated closely with hypoxic stress in M. japonicus [6], the immune response to laminarin stimulation in F. chinensis [15], E. coli LPS immune response to L. vannamei [17], salinity change in C. sapidus [20], the exposure to lead in yabby, Cherax destructor [21], acclimation to cadmium in crab, Eriocheir sinensis [22] and the defense against the infection of virus in shrimp, Penaeus stylirostris [23] and F. chinensis [24] suggesting that AK might play an important role in many biological events in crustaceans.

The Malaysian freshwater giant prawn Macrobrachium rose-nbergii is an important commercial species. However, infectious diseases especially, infectious hypodermal and hematopoietic necrosis virus (IHHNV) have affected aquaculture of M. rosenbergii enormously. Thus, research into freshwater prawn defense mechanisms is important to develop disease control strategies [17,24]. Yao et al. [17] reported that AK levels changed in prawn after immune stimulant, which suggested that the prawn immune response maybe correlated with the change of energy metabolism that was regulated by AK [25] and some studies showed that AK changed significantly after prawn were infected with virus [23,24] and bacteria [17]. However, the detailed func-tions and characterization of AK in prawn are poorly understood. To gain insight into the characterization of AK and its role in prawn, a full length cDNA of AK-1 (designated as MrAK-1) was identified from the M. rosenbergii transcriptome unigenes obtained by Illumina's Solexa sequencing technology. The expression profiles of AK mRNA in the muscle of M. rosenbergii after IHHNV challenge were studied and the enzyme activity of recombinant AK protein was assayed.

2. Materials and methods

2.1. Identification of M. rosenbergii arginine kinase-1

A full length MrAK-1 gene was identified from the M. rosenbergii transcriptome unigenes obtained by Illumina's Solexa sequencing technology. Briefly, unigenes obtained from the assembly of the Illumina Solexa short reads from the RNA sequencing of the muscle, gill and hepatopancrease transcriptomes of M. rosenbergii were mined for sequences which had been identified as arginine kinase 1 gene through BLAST homology search against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast).

2.2. Bioinformatics analysis

The Basic Local Alignment Tool (BLAST) program was used to search similar nucleotide and protein sequences to the MrAK-1 [26]. The open reading frame (ORF) and amino acid sequence of MrAK-1 was obtained by using DNAssit 2.2. Characteristic domains or motifs were identified using the PROSITE profile database [27]. Identity, similarity and gap percentages were calculated using FASTA program [28]. The N-terminal transmembrane sequence was determined by DAS transmembrane prediction program (http://www.sbc.su.se/wmiklos/DAS). Signal peptide analysis was done using the SignalP worldwide P server (http://www.cbs.dtu.dk). Pair-wise and multiple sequence alignment were analyzed using the ClustalW version 2 program [29]. The phylogenetic relationship of the MrAK-1 was determined using the neighbor-joining (NJ) method and MEGA 4.1 program [30].

2.3. M. rosenbergii and IHHNV challenge

Specific pathogen free (SPF) prawns (average body weight 10 g) were obtained from the Bandar Sri Sendayan, Negeri Sembilan, Malaysia. Prawns were maintained in flat-bottomed glass tanks (300 L) with aerated and filtered freshwater at 28 _ 1 ¯C in the laboratory. All prawns were acclimatized for 1 week before chal-lenged to IHHNV. A maximum of 15 prawns per tank were main-tained during the experiment.

For IHHNV induced mRNA expression analysis, the prawns were injected with IHHNV. Briefly, IHHNV infected prawn tail tissue, tested positive by nested PCR was homogenized in sterile 2% NaCl (1:10, w/v) solution and centrifuged in a tabletop centrifuge at 5000 rpm for 5 min at 4 ¯C. The supernatant was filtered through 0.45 mm filter and used for injecting (100 ml per 10 g prawn) the animals. Samples were collected before (0 h), and after injection (3, 6, 12, 24 and 48 h) and were immediately snap-frozen in liquid nitrogen and stored at _80 ¯C until the total RNA was isolated. Using a sterilized syringe, the haemolymph (0.2e0.5 ml per prawn) was collected from the prawn heart and immediately centrifuged at 3000 _ g for 10 min at 4 ¯C to allow haemocyte collection for total RNA extraction. All samples were analyzed in three duplications and the results are expressed as relative fold of one sample as mean _ standard deviation.

2.4. RNA extraction and cDNA synthesis

Total RNA was isolated from the tissues of each animal using TRI Reagent following manufacturer's protocol (Guangzhou Dongsheng Biotech, China). Total RNA was treated with RNase free DNA set (5 Prime GmbH, Hamburg, Germany) to remove the contaminating DNA. The total RNA concentration was measured spectropho-metrically (NanoVue Plus Spectrophotometer, GE Healthcare UK Ltd, England). First-strand cDNA was synthesized from total RNA by M-MLV reverse transcriptase (Promega, USA) following the

manufacturer's protocol with AOLP primer (5'GGCCACGCGTCGAC-TAGTAC(T)16(A/C/G)3').

2.5. Quantitative real-time PCR analysis of MrAK-1 mRNA expression

The relative expression of MrAK-1 in the hemocytes, pleopods, walking legs, eye stalk, gill, hepatopancreas, stomach, intestine, brain and muscle were measured by qRT-PCR. qRT-PCR was carried out using a ABI 7500 Real-time Detection System (Applied Bio-systems) in 20 μl reaction volume containing 4 μl of cDNA from each tissue, 10 μl of Fast SYBR Green Master Mix, 0.5 μl of each primer (20 pmol/μl) and 5 μl dH_2O. The qRT-PCR cycle profile was 1 cycle of 95 °C for 10s, followed by 35 cycles of 95 °C for 5s, 58 °C for 10s and 72 °C for 20s and finally 1 cycle of 95 °C for 15s, 60 °C for 30s and 95 °C. The same qRT-PCR cycle profile was used for the internal control gene, β-actin. The primers used in this study are presented in Table 1. β-actin primers were designed based on EST of 1357 bp (GenBank accession No. AY651918) from M. rosenbegrii. After the PCR program, data were analyzed with ABI 7500 SDS software (Applied Biosystems). To maintain consistency, the base-line was set automatically by the software. The comparative CT method (2^{-ddCT} method) was used to analyse the expression level of MrAK-1 [31]. All data were given in terms of relative mRNA expressed as means ± standard deviation. For comparison of rela-tive MrAK-1 mRNA expression, statistical analysis was performed using one-way ANOVA and mean comparisons were performed by Tukey's Multiple Range Test using SPSS 11.5 at 5% significant level.

2.6. In vitro expression construction

All of the cloning experiments were carried out according to Sambrook et al. [32] with slight modifications [33]. The primer set of MrAK-1 F5, MrAK-1 R6 was designed with the corresponding restriction enzyme sites for EcoRI and HindIII at the N- and C-termini for MrAK-1, respectively (Table 1) in order to clone the coding sequence into the expression vector, pMAL-c2X (New England Biolabs UK Ltd, United Kingdom). Using plasmid DNA of MrAK-1 as a template and Taq DNA polymerase (Invitrogen Bio-Services India Pvt. Ltd, Bangalore, India), PCR was carried out to amplify the coding sequence. The PCR product was purified using the QIAquick Gel Extraction Kit (QIAGEN India Pvt. Ltd., New Delhi, India). Then, both insert and vector were digested with the respective restriction enzymes. The ligated product was trans-formed into XL1 blue cells and the correct recombinant product (as confirmed by restriction enzyme digestion and sequencing) was transformed into competent E. coli BL21 (DE3) cells for protein expression.

2.7. In vitro purification of recombinant protein MrAK-1

Transformed E. coli BL21 (DE3) cells were incubated in ampi-cillin (100 μg/mL) Luria broth (LB) overnight. This culture was then used to inoculate 100 mL of LB broth in 0.2% glucose-rich medium with ampicillin at 37 °C until cell density reached 0.7 at OD_{600}. E. coli BL21 (DE3) harboring pMAL-c2x-MrAK-1 was induced for over expression with 1 mM isopropyl-β-thiogalactopyranoside (IPTG) and incubated at 15 °C for 4 h. Cells were harvested by centrifugation (4000 × g for 20 min at 4 °C). E. coli BL21 (DE3) uninduced culture was used as a negative control. Then the cells were resuspended in column buffer (TriseHCl, pH 7.4, 200 mM NaCl) and frozen at −20 °C overnight. After thawing on ice, cells were disrupted by sonication. The crude MrAK-1 fusion protein fused with maltose binding protein (MBP) was purified using pMAL™ protein fusion and purification system protocol (New England Biolabs UK Ltd, United Kingdom). Further the fusion protein was digested with thrombin and purified as described by Yao et al. [17], then the purity of the expressed enzyme was verified by 12% SDS-PAGE and the molecular weight of target protein was evaluated using protein molecular weight standards. Proteins were visualized by staining with 0.05% Coomassie blue R-250. The purified enzyme was kept at −80 °C until determination of enzy-matic activity.

2.8. Enzyme assay and protein determination

MrAK-1 activity was assayed using a modification of previous procedures [17, 34]. For the phosphate assay, the absorbance was measured using a UV-spectrophotometer. Appropriate controls consisted of assay samples lacking the prepared MrAK-1. Inorganic phosphate solutions were used to prepare a calibration curve. Briefly, the assay was performed using a stock solution prepared by combining 5.7 mM arginine, 6.6 mM magnesium acetate, complex acid-base indicator (consisting of 0.15% g/ml Thymol blue and 0.025% g/ml Cresol red), and 53.2 mg ATP, then adjusting the pH to 8.5 with 1 M NaOH. The reaction solution was pre-incubated for 10 min at 30 °C, prior to initiation of the enzymatic reaction upon the addition of 10 μl to 1 ml of reaction solution. The reaction progress was followed as a liner decrease in absorbance measured at 575 nm for 30 s using an analytic spectrophotometer Specord 200 UV VIS (Coslab, India). The slope was multiplied by the time to calculate the absorbance decrease. All measurements were carried out at 30 °C.

2.9. Measurement of optimum temperature, pH and MrAK-1 activity

For determination of the optimum temperature, aliquots of the purified recombinant MrAK-1 were incubated with standard buffer and assayed as described by Wang et al. [16] with the exception that the enzymatic activities were determined at different temperature gradients. For determination of optimum pH, the purified recombinant MrAK-1 preparation was added to various buffers with pH adjusted from 5.0 to 12.0, then incubated for 30 min at 30 °C prior to the recombinant MrAK-1 activity assay as described by Wang et al. [16].

To investigate the effect of some energy metabolism products on MrAK-1 activity, experiments were carried out [17] by supplementa-tion with glucose, ATP, α-ketoglutarate, citrulline, ornaline or glycerol

Table 1
Details of the primers used in this study.

Name	Target	Sequence (5'e3' direction)
MrAK-1 (F1)	qRT-PCR amplification	aagatcaagggcaacatcaacgcc
MrAK-1 (R2)	qRT-PCR amplification	aggcgctcttcatgaggttctcaa
β-actin (F3)	qRT-PCR internal control	accaccgaaattgctccatcctct
β-actin (R4)	qRT-PCR internal control	acggtcacttgttcaccatcggcatt
MrAK-1 (F5)	ORF amplification	GAGAGAGAATTCaccgcgagaagatgacacagatca (EcoRI)
MrAK-1 (R6)	ORF amplification	GAGAGAAAGCTTactcctgcttgctgatccacatct (HindIII)

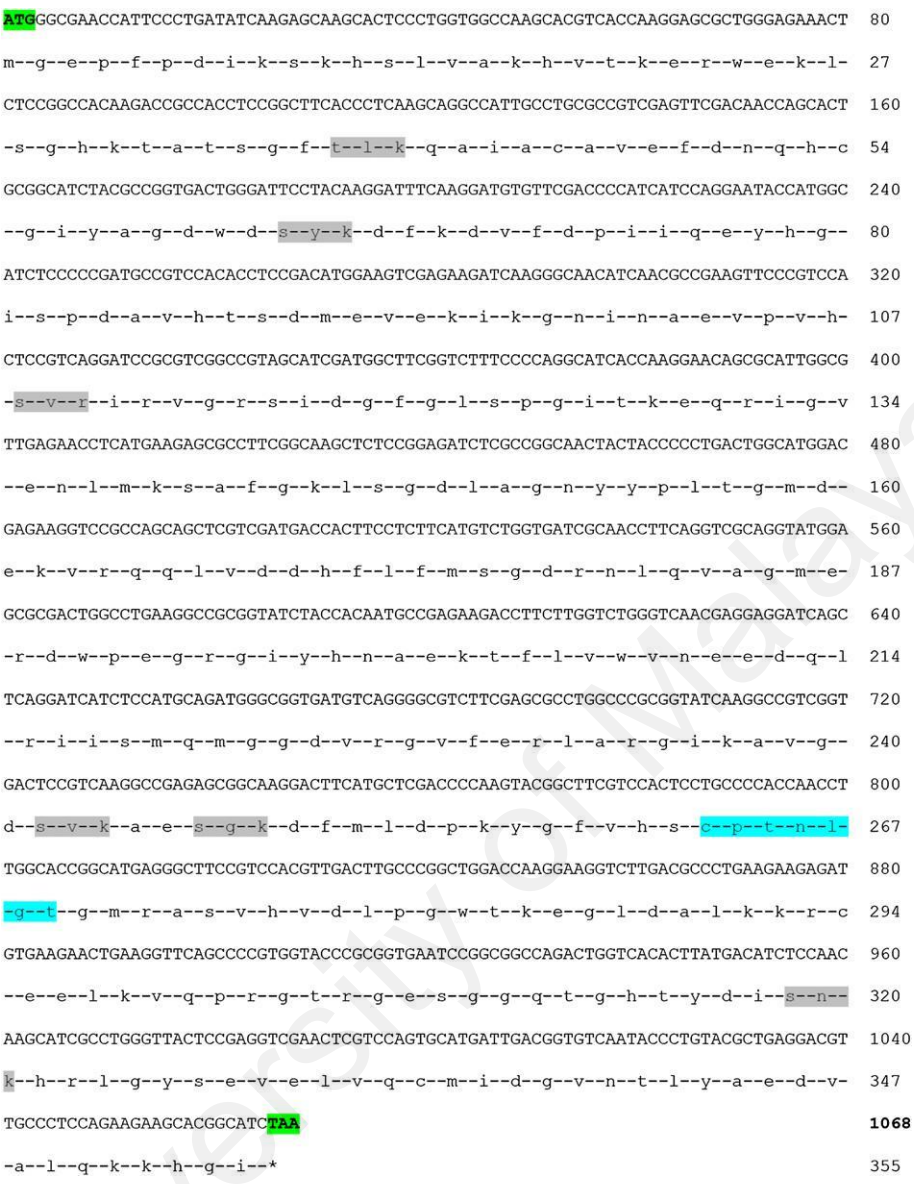

Fig. 1. Nucleotide and deduced amino acid sequences of M. rosenbergii arginine kinase-1 (MrAK-1). The nucleotide sequence is numbered from 5^{0} end, and the single letter amino acid code is shown below the corresponding codon. The start codon (ATG) and the end codon (TAA) highlighted in fluorescent green color. Potential ATP:guanido phospho-transferases active site highlighted in blue color. Six protein kinase C phosphorylation sites are highlighted in grey color. The termination code is marked with an asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the reaction buffer at a final concentration of 10, 50, 100 and 200 mM, respectively, in sealed glass vials containing 20 ml of enzyme in reaction buffer, pH 8.5. Each was with three replicates and the mixtures were withdrawn at appropriate times and immediately assayed for enzyme activity at 30 ¯C. Statistical analysis was performed using one-way ANOVA and mean comparisons were performed by Tukey's Multiple Range Test using SPSS 11.5.

3. Results

3.1. MrAK-1 sequence analysis

MrAK-1 was identified from the M. rosenbergii transcriptome unigenes obtained by Illumina's Solexa sequencing technology. The MrAK-1 sequences was submitted to GenBank under the accession number HQ191218. The nucleotide and deduced amino acid sequences of the MrAK-1 from rock bream are shown in Fig. 1.

MrAK-1 full length nucleotide consisted of 1068 base pair (bp) with an open reading frame (ORF) of 1065 bp. The ORF is capable of encoding a polypeptide of 355 amino acid (aa) with an estimated molecular mass of 40 kDa and a predicted isoelectric point (pI) of

Table 2
Pair-wise ClustalW analysis and comparisons of the deduced amino acid sequence of MrAK-1 with other known species. The GenBank accession number, gene and species details are given in the legend of Fig. 2.

Species	Amino acid length	Identity (%)	Similarity (%)	Gap (%)
N. denticulata I	355	96	98	0
N. denticulata II	355	85	93	0
L. salmonis	354	82	90	0
C. rogercresseyi	405	78	88	0
C. clemensi	357	78	88	0
M. ovata	389	52	64	1
C. gracilis	382	51	66	2

6.1. MrAK-1 aa sequence neither have a signal peptide nor a trans-membrane regions. The ExPASy motif analysis of MrAK-1 aa sequence contains a potential ATP:guanido phosphotransferases active site (Cys^{263}-Pro^{264}-Thr^{265}-Asn^{266}-Leu^{267}-Gly^{268}-Thr^{269}), 6 protein kinase C phosphorylation sites and 6 caesin kinase II phosphorylation sites (^{63}s-y-k-d^{66}; ^{89}s-ᴅ-m-e^{92}; ^{157}t-g-m-d^{160}; ^{247}s-g-k-d^{250}; ^{304}t-r-g-e^{307} and ^{327}s-e-v-e^{330}). A long ADP binding site is available in the aa sequence between 108 and 318. Another long phosphagen binding site is available in the aa sequence between 51 and 262. Additionally a small substrate specificity loop is also available in the aa sequence between 300 and 310.

The MrAK-1 amino acid identity and similarity percentages were calculated using FASTA program. We analyzed the MrAK-1 sequence identity with other seven homologous arginine kinase including arginine kinase 1 and 3 from cherry shrimp Neocaridina denticulate and argininie kinase from Lepeophtheirus salmonis, Caligus rogercresseyi, and C. clemensi and from euglena Monosiga ovata and Codonosiga gracilis (Table 2). Their respective aa sequence identity with MrAK-1 is as follows: 96%, 85%, 82%, 78%, 78%, 52% and 51%. In addition, MrAK-1 was aligned with the same seven homologous sequences using ClustalW multiple analyses (Fig. 2). The results revealed that C. rogercresseyi has the longest amino acid

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Macrobrachium rosenbergii    ----------------------------------------------------MGEPFPDIKS 10
Neocaridina denticulata I    ----------------------------------------------------MGEPFPDIKS 10
Neocaridina denticulata III  ----------------------------------------------------MGEPFPDIKS 10
Caligus rogercresseyi        MAEQQGFSHYFNSTTIKGRANSAKATLAVVGGAFAYFKFFKGNKTNLKAESAAPFPNIRS 60
Caligus clemensi             ----------------------------------------------------MSEVPPFPTIRS 12
Lepeophtherirus salmonis     ----------------------------------------------------MSDFPDIKS 9
Monosiga ovata               ------------------MEDTTYSLLLDIHERVKKIERNLGIKPGKKEAYG-AGFPSFNE 42
Codonosiga gracilis          -------------------------YAGIEARLSAIESSL---PGNGPSVSIDGYPRFPE 32
                                                                                  :* : .

Macrobrachium rosenbergii    K-HSLVAKHVTKERWEKLSGHKTATSGFTLKQAIACAVEFDNQHCGIYAGDWDSYKDFKD 69
Neocaridina denticulata I    K-HSLVAKHVTKERWDKLAAHKTATSGFTLKQAIACAVEFDNQHCGIYAGDWDSYKDFKD 69
Neocaridina denticulata III  K-HSLVAKYVTKDLWDKLSGIKTATSGFTLGKAIACAVDFDNQHCGIYAGDWDSYKDFKD 69
Caligus rogercresseyi        G-HSLVAKHVTKEKWDKLSGIETKTSGFTLAKAIACAVEFDDQHCGIYAGDEDSYADFAE 119
Caligus clemensi             S-HSLVAKHVTKEKWEKLSGISTKTSGFTLSKAIACAVEFDDQHCGIYAGDEDSYSDFAE 71
Lepeophtherirus salmonis     K-HSLVAKHVTKEKWDKLSKIVTKTSGFTLAKAIACAVEFDNQHCGIYAGDGDSYKDFGE 68
Monosiga ovata               KGRSLLKKYLTIDVYEQLKDKVTP-NGFTLERAIQSGVDNQDSGVGLYAGDEDAYTVFAP 101
Codonosiga gracilis          KCSSLLKKHLSNEVYLECAGRITP-SGFTLENVMQSGVDNFDSGVGCYAGDEESYTVFAP 91
                                ** *::: : : :     *  .**** ..: ..*:  :.  * **** ::*  *

Macrobrachium rosenbergii    VFDPIIQEYHG-ISPDAVHTSDMEVEKIKGNINA-EVPVHSVRIRVGRSIDGFGLSPGIT 127
Neocaridina denticulata I    VFDPIIQEYHG-ISPDAVHTSDMEVEKIKGNINT-EVPVHSVRIRVGRSIDGFGLSPGIT 127
Neocaridina denticulata II I VFDPIIQDYHG-IASDSKHTSDMDVGKLKGNINP-ECPVHSVRIRVGRNIDGFGLSPGIT 127
Caligus rogercresseyi        VFIPLICEYHG-VPPSFSHTSDMDSGKIKDNINP-DAPVHSTRIRVGRSIQGFGLSPGIT 177
Caligus clemensi             VFLPIICEYHG-VPPSFSHSSDMDSVKINGNINP-DAPVHSTRIRVGRSIQGFGLSPGIT 129
Lepeophtherirus salmonis     VFLPLICEYHG-LPADFKHTSDMDVSKIKGNVNS-EVPVHSCRIRVGRSIEGFGLSPGIT 126
Monosiga ovata               LFDAVIEDYHGGYKPTDKHVSDMDVSKVHGNPDPTGEDVVSTRIRVGRNIRGLGLPPGTT 161
Codonosiga gracilis          LFDRVIEDYHNGYKPTDKHVSDMDASKLHGSVDP--DYVISTRIRVGRNIRGLGLSPGIS 149
                             :*  :* :**.   .   * ***:  *::.. :.    * * ******.* *:**.** :

Macrobrachium rosenbergii    KEQRIGVENLMKSAFGKLSGDLAGNYYPLTGMDEKVRQQLVDDHFLFMSGDRNLQVAGME 187
Neocaridina denticulata I    KEQRVGVENLMKSAFAKLSGDLSGNYYPLTGMDEKVRQQLVDDHFLFMSGDRNLQVAGME 187
Neocaridina denticulata III  RDQRLGVENLMKHAFQKLTGDLAGNYYPLVGMEESVRQQLVDDHFLFMSGDPNLQVAGME 187
Caligus rogercresseyi        KQQRLDVESLMKTAFGNLKGDLSGSYFPLLGMDEATRTQLVDDHFLFVSGDRNLKVGGME 237
Caligus clemensi             QQQRLDVESLMKTAFKNLQGDLSGSYYPLLGMEEATRKQLVDDHFLFVSGDRNLKVGGME 189
Lepeophtherirus salmonis     QEQRVGVESLMKNAFKKLPEGLNGNYYPLIGMDEKVRQQLVDDHFLFVSGDRNLTVAGME 186
Monosiga ovata               RSQRREVERVVTKGLSTFTGDLKGKYYPLGKMTEAERKQLVEDHFLFKKGDRFLESAGAN 221
Codonosiga gracilis          RAQRRVSESILVDALSKVDGDLEGKYYSLGNMSEEDRKQLVADHFLFKKGDRFLQSAGAN 209
                             : **   * ::  .: ..  .* *.*:.*  * *  * *** ***** .** *  .* :

Macrobrachium rosenbergii    RDWPEGRGIYHNAEKTFLVWVNEEDQLRIISMQMGGDVRGVFERLARGIKAVGDSVKAES 247
Neocaridina denticulata I    RDWPEGRGIFHNAEKTFLVWVNEEDQLRIISMQKGGDVRGVFERLARGIKAVGDSVKAES 247
Neocaridina denticulata III  REWPEGRGIFHNKDKTFLVWVNEEDQLRIISMQKGGDVKAVFERLARGIQAVGDSVKSES 247
Caligus rogercresseyi        RDWPEGRGIFHNKEKTFLTWVNEEDQLRIISMQKGGDVKGVLERLAKGIKAVGDSVKAES 297
Caligus clemensi             RDWPEGRGIFHNKEKTFLTWVNEEDQLRIISMQKGGDVKGVFERLARGINAVGDSVKAES 249
Lepeophtherirus salmonis     RDWPEGRGIFHNESKTFLVWVNEEDQLRIISMQKGGDVKGVFERLAKGIKAVQDSVKAES 246
Monosiga ovata               RDWPEGRGIFHNEAKSFLVWVNEEDQMRIISMQPGGNVKEVFERLARGINAAEGVVKT-S 280
Codonosiga gracilis          RDWPESRGIFHNNEKTFLVWVNEEDQARIISMEQGGDVKGIFERLSRGIAAIEKGVQA-S 268
                             *:***.***:**  *:**.******* *****: **:*: ::***::** *    *:: *

Macrobrachium rosenbergii    GKDFMLDPKYGFVHSCPTNLGTGMRASVHVDLPGWTKEGLDALKKRCEELKVQPRGTRGE 307
Neocaridina denticulata I    GKDFMLDSKYGYVHSCPTNLGTGMRASVHVDLPGWTKEGLEALKKRCEELKVQPRGTRGE 307
Neocaridina denticulata III GKEFAFDEKYGFIHSCPTNLGTGMRASVHVDLPGWTKEGLDALKKRCEELKVQPRGTRGE 307
Caligus rogercresseyi        GKEFLLDPKLGYLHSCPTNLGTGMRASVHIDLPGWTKEGLPALKKRCEELSLQPRGTRGE 357
Caligus clemensi             GKEFLLDPKLGYLHSCPTNLGTGMRASVHIDLPGWTKEGLPALKKRCEELALQPRGTRGE 309
Lepeophtherirus salmonis     GKDFMLDPQLGYLHSCPTNLGTGMRASVHVDLPGWTKEGVDHLKKRCEELALQPRGTRGE 306
Monosiga ovata               GYEFSYNDHLGFIHSCPTNCGTGMRASVHVKLPNVSKH--ANFHAWCDKLRLQPRGIHGE 338
Codonosiga gracilis          GYEYAYNDHLGYIHSCPTNCGTGMRASVHVKLPNVSKH--PDFKNWCEKLRLQPRGIHGE 326
                             * ::  : : *::****** *********:.**. :*.    ::  *::* :**** :**


Macrobrachium rosenbergii    SGGQTGHTYDISNKHRLGYSEVELVQCMIDGVNTLYAEDVALQKKHGI-------- 355
Neocaridina denticulata I    SGGQTGHTYDISNKHRLGYSEVELVQCMIDGVNTLYAEDVALQKKHGI-------- 355
Neocaridina denticulata III  SGGQTGVTYDISNKHRLGYSEVELVQCMIDGVNALHEEDIKLQKKHGL-------- 355
Caligus rogercresseyi        SGGQTGVTYDISNKHRLGYSEVQLVQTMIDGVNTLFKEDLELKKKHSL-------- 405
Caligus clemensi             SGGQTGVTYDISNKHRLGYSEAQLVQTMIDGVNTIYKEDLELKKKHGL-------- 357
Lepeophtherirus salmonis     SGGQTGHTYDISNKHRLGYSEIQLVQTMIDGVNTLYKEDLEFQKKHGM-------- 354
Monosiga ovata               HSETEGGVYDISNKERLGKSEVELVQTMIDGVTFLINAEKALAAG---KQPPAL-- 389
Codonosiga gracilis          HSESEGGVYDISNKERLGKSEVQLVQTMIDGVTVLIDAEKSLESKGTIPLPSKLLQ 382
                               .   * .******.*** ** :*** *****. :   :  :
```

Fig. 2. Multiple sequence alignments of MrAK-1 with seven other homologous arginine kinase amino acid sequences. Arginine kinase 1 of cherry shrimp N. denticulata I (BAH56608), argininie kinase 3 of N. denticulata III (BAH56610), arginine kinase of sea louse L. salmonis (ACO11785), C. rogercresseyi (ACO11152), C. clemensi (ACO15761), euglena M. ovata (ABN49969) and C. gracilis (ABN49968) are shown. Asterisk marks indicates identical amino acids and numbers to the right indicate the aa position of arginine kinase in the corresponding species. Conserved substitutions are indicated by (:) and semi-conserved substitutions are indicated by (.). Deletions are indicated by dashes. GenBank accession numbers for the amino acid sequences of arginine kinase given in the parentheses.

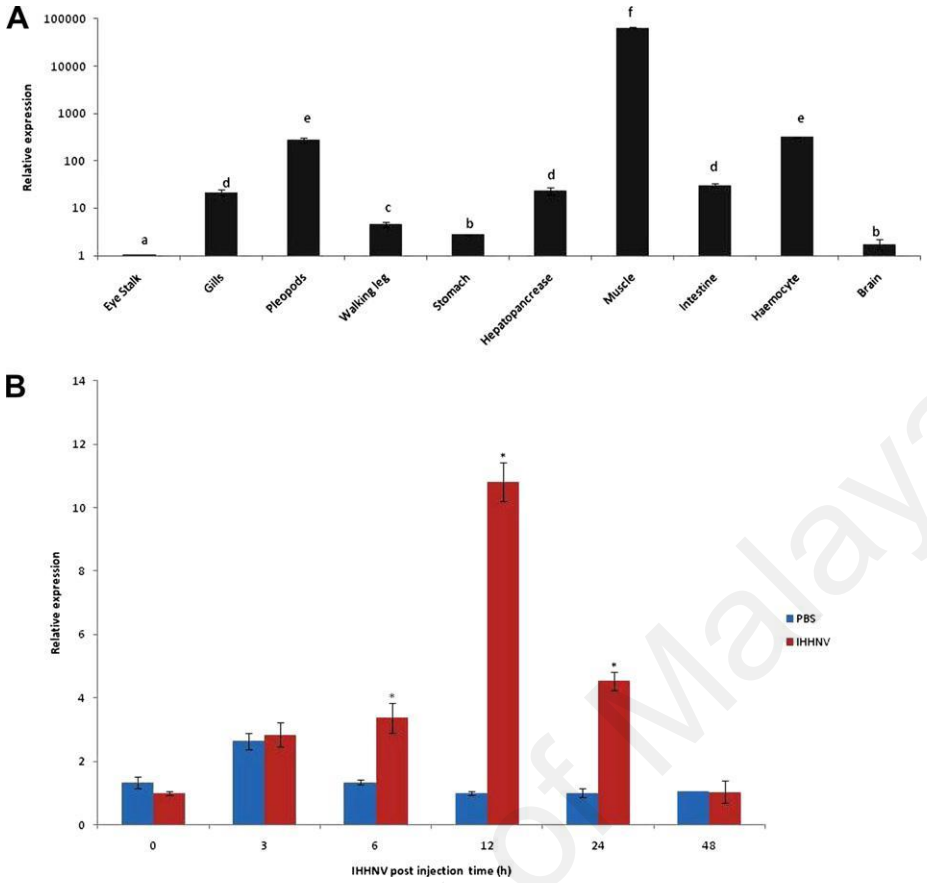

Fig. 3. Gene expression patterns of MrAK-1 by qRT-PCR. 3A: Tissue distribution of MrAK-1 in different tissues of M. rosenbergii. Data are expressed as a ratio to MrAK-1 mRNA expression in eye stalk. The different alphabets are statistically significant at $P < 0.05$ level by one-way ANOVA and Tukey's Multiple Range Test. 3B: The time course of MrAK-1 mRNA expression in muscle at 0, 3, 6, 12, 24, and 48 h post-injection with IHHNV. Data are expressed as a ratio to MrAK-1 mRNA in sample from unchallenged control group. The significant difference of MrAK-1 expression between the challenged and the control group were indicated with asterisks.

sequence (405 aa), even though conserved motifs were observed among the sequences, the length of the amino acids varied from species to species.

Phylogentic analysis was performed using full length amino acid sequence of MrAK-1 and other 7 representatives [argininie kinase 1 of cherry shrimp Neocardina denticulata (BAH56608), argininie kinase 3 of N. denticulata (BAH56610), arginine kinase of sea louse Lepeoph-therirus salmonis (ACO11785), Caligus rogercresseyi (ACO11152), Caligus clemensi (ACO15761), euglena Monosiga ovata (ABN49969) and Codonosiga gracilis (ABN49968)]. The results of phylogenetic tree showed three distinct clusters of decapoda, copepoda and choano-flagellida (data not shown). MrAK-1 closely clustered with the homologous AK-1 from cherry shrimp Neocardina denticulata and formed a sister group with AK-3 from N. denticulata and finally clus-tered together with the AK from copepods. The results of the phylo-genetic tree are agreeable with the traditional taxonomy.

3.2. MrAK-1 mRNA expression in tissues

The mRNA transcripts of MrAK-1 could be detected by quanti-tative real-time RT-PCR mainly in muscle (Fig. 3A). The largest quantity of MrAK-1 was shown in muscle, then haemocyte, pleo-pods, intestine, hepatopancrease and gills, and the expression of MrAK-1 in walking leg, stomach, brain and eye stalk was very low. Further analysis results showed that MrAK-1 mRNA expression was significantly higher ($P < 0.05$) in the muscle. Therefore, muscle tissue was selected to investigate the temporary expression of MrAK-1 gene after IHHNV challenge.

3.3. MrAK-1 mRNA expression in muscle after IHHNV challenge

To analyze the expression profile of M. rosenbergii MrAK-1 during infection, prawns were infected with IHHNV and the muscle was analyzed by real-time PCR (Fig. 3B). The levels of MrAK-1 mRNA transcripts sharply increased until 12 h post-injection, a slight

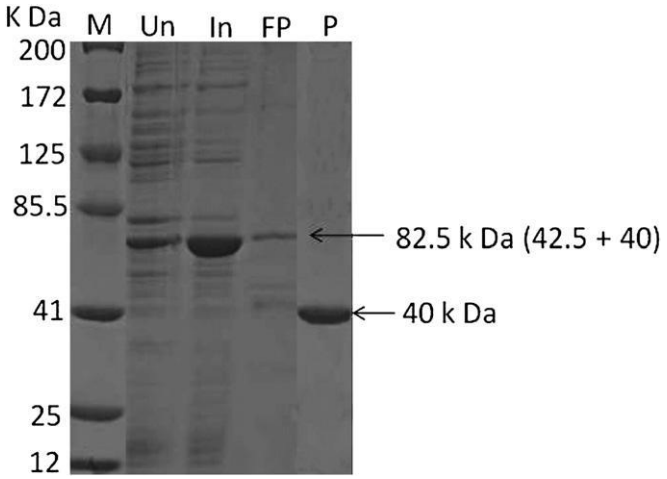

Fig. 4. Expression and purification of the recombinant MrAK-1 protein. Protein samples were separated by SDS-PAGE and stained with Coomassie brilliant blue. M, protein marker; Un, before induction with IPTG; In, after IPTG induction; PF, purified fusion protein and P, purified recombinant protein.

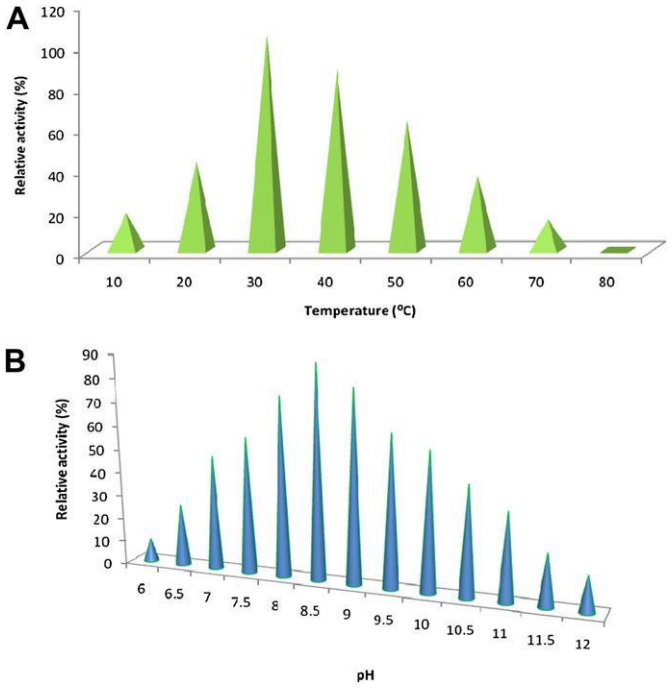

Fig. 5. The effect of temperature and pH on the activity of expressed MrAK-1. 5A: The assay systems were incubated for 30 min in the various temperatures, then added the enzyme to the system determining the enzyme activity. 5B: The assay systems were incubated at 30 °C for 5 min before the enzyme activity was determined. The pH 6.0e6.5 was sodium citrateecitrate buffer, pH 7.0e10.0 were TriseHCl buffer and pH 10.0e12.0 were glycine-NaOH buffer.

decrease of MrAK-1 mRNA expression appeared at 24 h and at 48 h the expression was reached almost near to the basal level. Significant differences ($P < 0.05$) in expression were found at 6,12 and 24 h post-injection between the IHHNV challenged and the PBS injected control group.

3.4. Expression and purification of recombinant MrAK-1 protein

The putative mature MrAK-1 molecule cDNA was expressed in E. coli cells after cloning the cDNA into the EcoRI and HindIII restriction sites of pMAL-c2x-MrAK-1 expression vector, IPTG driven expression of MrAK-1 was done in E. coli BL 21 (DE3) cells. The recombinant MrAK-1 was purified from the supernatant of induced cells. Fig. 4 (lane PF) shows the result of SDS-PAGE of the recombinant MrAK-1 along with fusion protein, the recombinant enzyme gave a major single band with molecular mass around 82.5 kDa (42.5 kDa for MBP and 40 kDa for MrAK-1 enzyme), suggesting that the enzyme is purified enough to determine its characterization. After digestion by thrombin, the recombinant MrAK-1 protein showed a single band with molecular weight about 40 kDa (Fig. 4).

3.5. Optimum temperature and pH for MrAK-1 activity

The optimum temperature was determined using the purified recombinant MrAK-1 protein along with the buffer. The assay results showed that the optimum temperature for MrAK-1 activity is 30 °C (Fig. 5A). And also we used the purified recombinant MrAK-1 protein along with various pH (5.0e12.0) buffers to determine the optimum pH. During the assay the recombinant MrAK-1 protein showed the highest activity in the synthesis direction of phos-phoarginine and ADP at pH 8.5 (Fig. 5B), and this is taken into account as optimum pH for MrAK-1 activity.

3.6. MrAK-1 activity assay

The results of MrAK-1 enzyme activity and characterization assay are presented in Fig. 6. The purified recombinant MrAK-1 was used to determine its characterization. Following treatment with different energy metabolism products, MrAK-1 activity showed some changes (Fig. 6). MrAK-1 activity did not show significant variation after supplementation with 10 mM glucose. However, mrAK-1 activity decreased significantly when glucose concentration was higher than 50 mM ($P < 0.05$) and almost all MrAK-1 activity was lost after treatment with 200 mM glucose ($P < 0.05$).

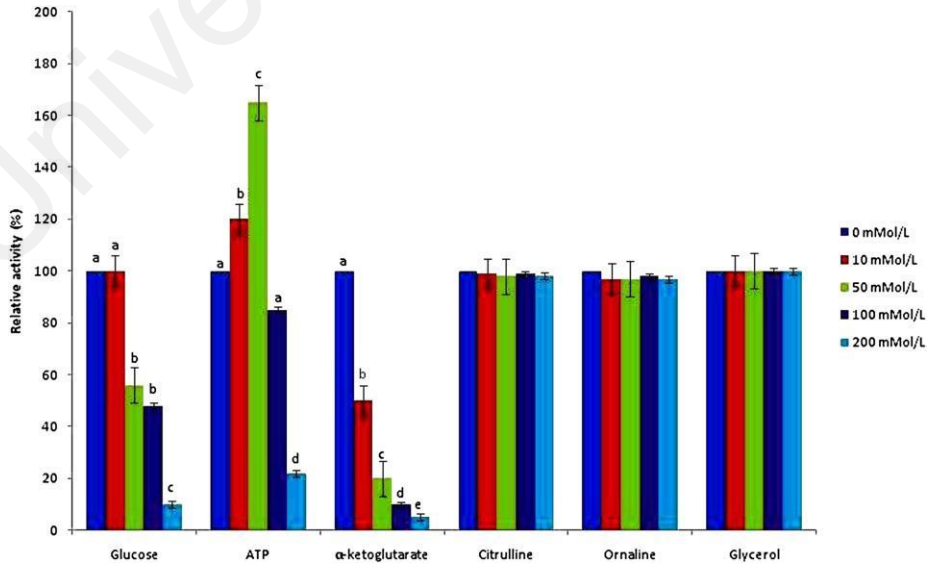

Fig. 6. Effect of different energy metabolism products, include 10, 50, 100 and 200 mM glucose, ATP, a-ketoglutarate, citrulline, ornaline and glycerol, on MrAK-1 activity. Each test was carried out in sealed glass vials containing 20 ml of enzyme in reaction buffer with different concentration of energy metabolism products at 30 °C. The data were analyzed by using SPSS 11.5. software. The significant difference of MrAK-1 activity between the treatment and the control group of each metabolic product were indicated with alphabets. The different alphabets are statistically significant at $P < 0.05$ level by one-way ANOVA and Tukey's Multiple Range Test.

MrAK-1 activity was significantly ($P < 0.05$) higher than that of the control after treatment with 10 mM and 50 mM ATP, however, MrAK-1 activity dropped significantly following incubation with 200 mM ATP. The enzyme activity was inhibited by a-ketoglutarate at the concentration between 10 and 200 mM, and the inhibition was in direct ratio to the a-ketoglutarate concentration ($P < 0.05$). MrAK-1 activity did not show significant variation after incubated with 10e200 mM citrulline, ornaline and glycerol.

4. Discussion

AK plays a key role in the coupling of energy production and utilization in animals [35]. Several AK from shrimps such as Mar-supenaeus japonicus [6], Fenneropenaeus chinensis [15], Meta-penaeus ensis [16], Litopenaeus vannamei [17,26], Penaeus japonicus [36], P. aztecus [37] and P. monodon [38] were purified, cloned or identified. In this study, a full-length cDNA of MrAK-1 was identi-fied and sequenced from M. rosenbergii. An ORF of 1065 bp that coded for 355 aa was obtained (Fig. 1), which showed a high degree of similarity to known arginine kinase in shrimps and to other predicted proteins (Fig. 2 and Table 2). This finding supported a number of previous studies that provided evidence for an excellent conservation of both primary and secondary structures amongst phosphagen kinases [15,17,37,39]. Three conserved domains such as ATP:guanido phosphotransferases domain, ADP binding domain and phosphagen binding domain were predicted in the sequence, which corresponded to other AK [17,37].

A phylogenic tree was constructed based on the amino acid sequence of MrAK-1 and other homologous sequences from decapoda, copepoda and choanoflagellida. The genetic distance among the species taken for phylogenetic analysis is 0.1. In the phylogenic tree, MrAK-1 was clustered and had high similarity with AK-1 from N. denticulata, together with the structural features, indicating that MrAK-1 should be an isomer of arginine kinase. Although MrAK-1 and AK from the copepoda and choanoflagellida, their sequences and structural characteristic was disparate, and clustered separately in the phylogenic tree, it may be due to their taxonomical differentiation as reported by Wang et al. [16].

In general, most research about AK has focused on the AK characterization and functions in muscle or gill in crustaceans [17,21,38e40]. In the present study, MrAK-1 transcripts were observed in most examined tissues in M. rosenbergii with the most predominant expression being in muscle (Fig. 3 A), which is similar with the AK in other shrimp species [15,17,38e41]. Previous research indicated that the variation of AK expression might be related to some external factors in invertebrates. It was reported that AK expression and activity change correlated closely with the salinity change in crabs [21,40]. AK concentration and specific activity showed significant variation after exposure to toxic and hypoxia stress in shrimps [6], shellfish lobster [21] and crabs [20]. Furthermore, AK also changed in shrimp after immune stimulation. For example, AK protein changed significantly in shrimp F. chinensis hemolymph after injection with laminarin [15]. Astrofsky et al. [23] demonstrated that AK transcript expression changed in shrimp P. stylirostris and F. chinensis [24] after white spot virus infection. In this study, the mRNA expression of MrAK-1 was detected by qRT-PCR, which is the first report of it in shrimp muscle after IHHNV challenge (Fig. 3B). At 12 h after IHHNV challenge, the increased MrAK-1 expression in muscle may due to the up-regulation of gene expression, proliferation or recruitment of MrAK-1 expressing cells in the tissue. The pattern gene expression after IHHNV challenge, showing that MrAK-1 might be involved in a transient systemic immune response to the stimulation inflicted by the immune challenge and the invasion of heterogenous substances as reported by Yao et al. [17].

This MrAk-1 sequence was validated by the pMAL-c2x-MrAK-1 expression vector and expressed in E. coli as fusion protein. Recombinant MrAK-1 was purified to homogeneity using pMAL^{TM} protein fusion and purification system followed by digestion with thrombin. The molecular mass of protein was about 40 kDa on 12% SDS-PAGE gel (Fig. 4), similar to the earlier reported AK of Abe et al. [6], Holt and Kinsey [9], Yao et al. [15], Wang et al. [16], Yao et al. [17], Tada and Suzuki [18], Kinsey and Lee [20], Astrofsky et al. [23] and Wang et al. [24].

The optimum temperature and pH for MrAK-1 enzyme activity is 8.5 and 30 $^{-}$C respectively (Fig. 5 A and B). This optimum levels are quite different from the optimum pH (7.5e8.0) reported by Qing-Yun et al. [41] for monomeric AK from scallop, but very similar to that of greasyback shrimp M. ensis [16], white shrimp L. vannameii [17] and American cockroach [42].

The results of enzyme activity showed that the MrAK-1 enzyme activity is enhanced when adding low concentration of ATP (10e50 mM), and the activity is reduced when increasing the concentration of ATP (100e200 mM). A similar result was demon-strated by Watts et al. [43] in Trypanosoma cruzi. MrAK-1 activity was inhibited significantly by 10 mM a-ketoglutarate and almost all of the MrAK-1 activity was lost increasing concentrations, which may suggest it can combine with the active site of the enzyme. It has been indicated that MrAK-1 activity may be correlated with the tricarboxylic acid cycle and carbohydrate metabolism [17]. The presence of 10e200 mM citrulline, ornaline and glycerol did not decrease MrAK-1 activity significantly, suggesting that MrAK-1 activity was not affected significantly by the main products of arginine catabolism and the primarily products of triglyceride glycerol catabolism. However, further research is necessary on the detailed mechanism.

Conclusively, MrAK-1 was identified from the transcriptome database of M. rosenbergii. MrAK-1 belonged to the conserved ATP:guanido phosphotransferases family. MrAK-1 was predomi-nantly expressed in most tissues in M. rosenbergii with the highest level in muscle and the lowest level in eyestalk. The expression of MrAK-1 in muscle changed rapidly and dynamically in response to IHHNV challenge, which suggested that MrAK-1 expression corre-lated closely with the shrimp immune response. We successfully expressed the MrAK-1 gene from M. rosenbergii through an E. coli expression vector and acquired a highly purified functional enzyme. Some characterizations of the enzyme have been uncov-ered, however, most details of the active site and catalytic mecha-nism of the enzyme are still unclear, and the findings of this study may lead to further research.

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APPENDIX A: Purified *Mr*MSTN PCR Amplified Products for sequencing

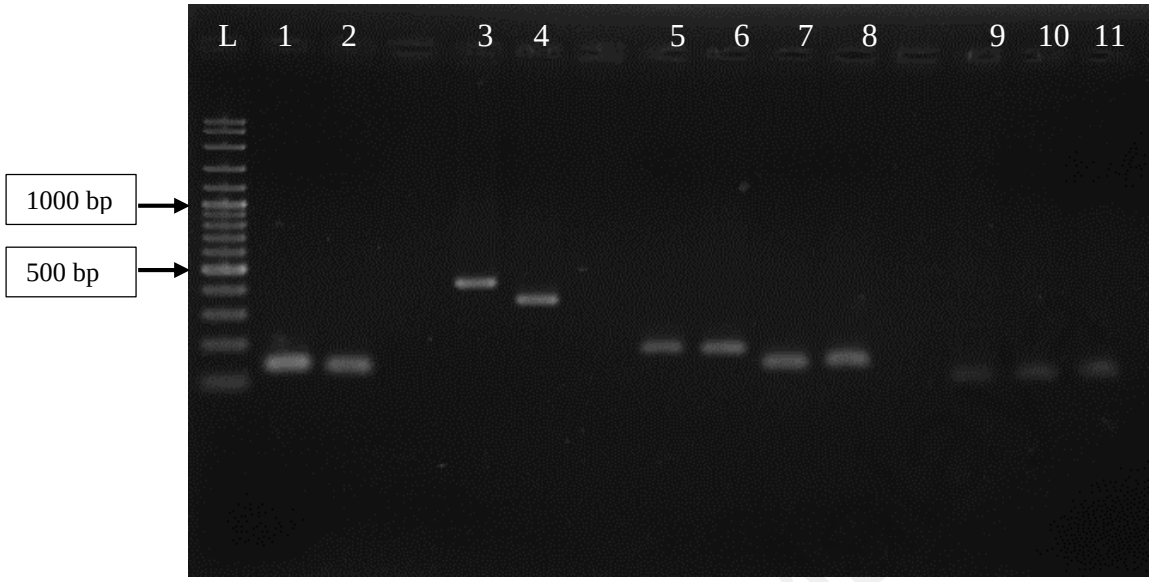

Figure A : Figure represents 1% (w/v) agarose gel of purified *Mr*MSTN cloned pcr product for sequencing :- Lane L: Vivantis 100bp ladder, Lane 1- 11 Amplified product of respective primer : Lane 1 - primer A1; Lane 2- Primer A2; Lane 3- Primer B1; Lane 4- Primer B2; Lane 5- Primer C1; Lane 6- Primer C2; Lane 7- Primer C3; Lane 8- Primer C4; Lane 9- Primer D1; Lane 10- Primer D2; Lane 11- Primer D3.

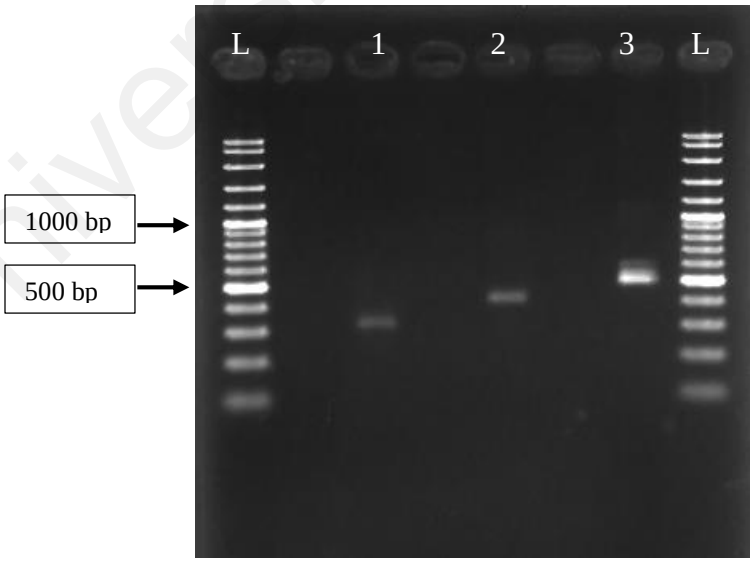

Figure B: Figure represents 1% (w/v) agarose gel of purified *Mr*MSTN RACE-PCR product for sequencing: - Lane L: Vivantis 100bp ladder, Lane 1- 3 Amplified product of 5' and 3' RACE PCR: Lane 1 – 5' RACE PCR; Lane 2- 3' RACE PCR A; Lane 3- 3' RACE PCR B.

APPENDIX B: IHHNV positive testing in IHHNV challenged *M. rosenbergii*

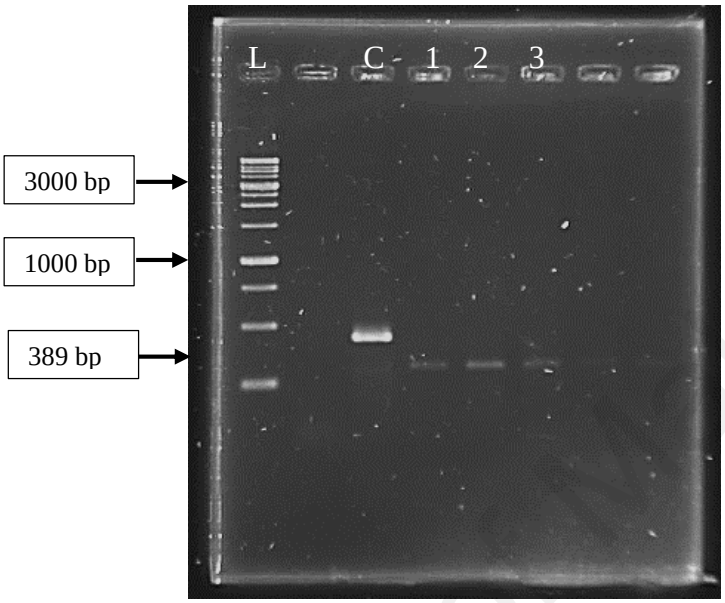

Figure A: Figure represents 1% (w/v) agarose gel of IHHNV-positive testing randomly in IHHNV challenged *M. rosenbergii* at time point: - Lane L: Promega 1 Kb ladder, Lane C: WSSV and IHHNV (389 bp) control; Lane 1: 48h, Lane 2: 12 hour; Lane 3: 3 hour

APPENDIX C: Histology

1. Staining Method of Hematoxylin and Eosin (H&E)

Staining Solution	Time
Xylene I	3 minutes
Xylene II	3 minutes
100% Alcohol	3 minutes
95% Alcohol	3 minutes
80% Alcohol	3 minutes
70% Alcohol	3 minutes
Running water	3 minutes
Erlich Hematoxylin	10 minutes
Quick Dip in Acid-Alcohol	Quick dip
Running water	3 minutes
Water and $LiCo_3$	2 or 3 times (1-2 Seconds)
Eosin	3 minutes
95% Alcohol	3 minutes
100% Alcohol	3 minutes
Xylene III	3 minutes
Xylene IV	3 minutes

2. Preparation of Davidson Fixative (DF)

Formaldehyde	2 Parts
Glycerol	1 Part
Glacial Acetic Acid	1 Part
Absolute Alcohol	3 Parts
Sea Water	3 Parts

APPENDIX D: qRT-PCR ($2^{-\Delta\Delta CT}$) for tissue distribution and One-way ANOVA

Myo 4	Eyes	Muscle	Stomach	hepatopancrease	Heart	Gill	Intestine	Haemocyte	Pleopod
	21.687	25.141	24.434	28.562	27.063	22.263	29.116	28.926	29.7
	21.438	25.472	24.342	28.821	27.1	22.153	29.484	28.244	30
	21.935	25.972	24.01	28.129	27.577	22.101	29.176	28.313	29.484
	21.68666667	**25.52833333**	**24.262**	**28.504**	**27.24667**	**22.17233**	**29.25866667**	**28.49433333**	**29.728**
EF	24.154	17.129	20.173	30.304	20.451	19.482	21.098	22.67	27.112
	24.561	17.602	20.815	30.658	20.448	19.685	21.86	22.76	27.646
	24.156	17.491	20.794	30.606	20.147	19.944	21.784	22.125	27.123
	-2.46733333	8.399333333	4.089	-1.8	6.795667	2.690333	8.160666667	5.824333333	2.616
	-2.87433333	7.926333333	3.447	-2.154	6.798667	2.487333	7.398666667	5.734333333	2.082
	-2.46933333	8.037333333	3.468	-2.102	7.099667	2.228333	7.474666667	6.369333333	2.605
	-2.60366667	**-2.603666667**	**-2.60367**	**-2.603666667**	**-2.60367**	**-2.60367**	**-2.603666667**	**-2.603666667**	**-2.603666667**
	-0.13633333	-11.003	-6.69267	-0.803666667	-9.39933	-5.294	-10.76433333	-8.428	-5.219666667
	0.270666667	-10.53	-6.05067	-0.449666667	-9.40233	-5.091	-10.00233333	-8.338	-4.685666667
	-0.13433333	-10.641	-6.07167	-0.501666667	-9.70333	-4.832	-10.07833333	-8.973	-5.208666667
	1.099108136	2052.263127	103.4412	1.745531832	675.2759	39.23312	1739.350942	344.4140054	37.26286422
	0.828936407	1478.583496	66.28758	1.365724671	676.6816	34.08346	1025.6575	323.5847932	25.73512111
	1.097585504	1596.835518	67.25952	1.41584827	833.6704	28.48242	1081.136774	502.5070407	36.97982947
Average	**1.008543349**	**1709.22738**	**78.99609**	**1.509034924**	**728.5426**	**33.933**	**1282.048405**	**390.1686131**	**33.32593827**
Std. Dev	**0.001524613**	**302.9043213**	**21.17564**	**0.206339973**	**91.04603**	**5.376925**	**397.0059131**	**97.84378241**	**6.575363565**

ANOVA - Tissue Distribution

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9724176.163	8	1215522.020	40.858	.000
Within Groups	535496.259	18	29749.792		
Total	10259672.422	26			

		Subset for alpha = .05				
REPLICATE	N	1	2	3	4	5
1.00	3	********				
4.00	3	********				
9.00	3	********				
6.00	3	********				
3.00	3	********				
8.00	3		********			
5.00	3			********		
7.00	3				********	
2.00	3					********
Sig.		.624	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

APPENDIX E: qRT-PCR ($2^{-\Delta\Delta CT}$) for different life stages and One-way ANOVA

Myo 4	Larvae	Post-Larvae	Juvenile	Adult
	30.345	28.294	26.2	25.347
	30.244	28.398	26.207	25.722
	30.507	28.043	26.186	25.51
	30.36533	**28.245**	**26.19767**	**25.52633**
E.Factor	23.852	26.156	20.086	20.138
	23.709	26.141	20.11	20.497
	23.941	26.014	20.024	20.583
	6.513333	2.089	6.111667	5.388333
	6.656333	2.104	6.087667	5.029333
	6.424333	2.231	6.173667	4.943333
	0	**2.141333333**	**2.141333**	**2.141333**
	-6.51333	0.052333333	-3.97033	-3.247
	-6.65633	0.037333333	-3.94633	-2.888
	-6.42433	-0.089666667	-4.03233	-2.802
	91.35003	0.964375341	15.67435	9.493894
	100.8686	0.974454458	15.41575	7.402435
	85.88494	1.064124289	16.36264	6.974066
Average	**92.70119**	**1.000984696**	**15.81758**	**7.956799**
Std. Dev	**7.582659**	**0.054912233**	**0.489422**	**1.348285**

ANOVA -Different Life Stage

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	695.710	3	231.903	184.984	.000
Within Groups	10.029	8	1.254		
Total	705.739	11			

MrMSTN

Tukey HSD[a]

LIFESTAG	N	Subset for alpha = .05			
		1	2	3	4
2.00	3	********			
4.00	3		********		
3.00	3			********	
1.00	3				********
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

APPENDIX F1 – qRT-PCR ($2^{-\Delta\Delta CT}$) for IHHNV Challenged *M.rosenbergii* (*Mr*MSTN) and One-way ANOVA

IHHNV Infected	0 Hour	3 Hour	6 Hour	12 Hour	24 Hour	48 Hour
Myo	22.363	24.016	25.646	24.063	23.812	23.076
	22.289	24.474	25.891	24.14	23.404	23.291
	22.161	24.336	25.606	24.009	23.686	23.156
	22.271	24.27533	25.71433	24.07067	23.634	23.17433
Elongation Factor	15.239	15.701	16.054	15.373	15.25	15.749
	15.324	15.788	16.001	15.465	15.339	15.342
	15.374	15.787	16.111	15.744	15.236	15.58
	7.032	8.574333	9.660333	8.697667	8.384	7.425333
	6.947	8.487333	9.713333	8.605667	8.295	7.832333
	6.897	8.488333	9.603333	8.326667	8.398	7.594333
	6.958667	6.958667	6.958667	6.958667	6.958667	6.958667
	-0.07333	-1.61567	-2.70167	-1.739	-1.42533	-0.46667
	0.011667	-1.52867	-2.75467	-1.647	-1.33633	-0.87367
	0.061667	-1.52967	-2.64467	-1.368	-1.43933	-0.63567
	1.052145	3.064532	6.50553	3.338037	2.685765	1.381913
	0.991946	2.885191	6.748967	3.131817	2.525087	1.832314
	0.958157	2.887191	6.253512	2.581125	2.711955	1.553656
Average	1.000749	2.945638	6.50267	3.016993	2.640936	1.589294
Std Deviation	0.047609	0.10297	0.24774	0.391302	0.101179	0.227306

APPENDIX F2 – qRT-PCR ($2^{-\Delta\Delta CT}$) for PBS Challenged *M.rosenbergii* (*Mr*MSTN) And One-way ANOVA

PBS Injected	0 Hour	3 Hour	6 Hour	12 Hour	24 Hour	48 Hour
*Mr*MSTN	22.039	23.379	23.048	23.234	22.538	23.511
	22.27	23.284	23.176	23.232	22.588	23.578
	22.166	23.393	23.226	23.273	22.029	23.257
	22.15833	23.352	23.15	23.24633	22.385	23.44867
EF	15.629	16.707	16.532	16.391	15.585	16.577
	15.237	16.967	16.565	16.361	15.647	16.837
	15.519	16.914	16.75	16.191	15.607	16.866
	6.529333	6.645	6.618	6.855333	6.8	6.871667
	6.921333	6.385	6.585	6.885333	6.738	6.611667
	6.639333	6.438	6.4	7.055333	6.778	6.582667
	6.489333	6.489333	6.489333	6.489333	6.489333	6.489333
	-0.04	-0.15567	-0.12867	-0.366	-0.31067	-0.38233
	-0.432	0.104333	-0.09567	-0.396	-0.24867	-0.12233
	-0.15	0.051333	0.089333	-0.566	-0.28867	-0.09333
	1.028114	1.113936	1.093283	1.288775	1.240281	1.303448
	1.349103	0.930235	1.068559	1.315855	1.188109	1.088494
	1.109569	0.965044	0.939957	1.480413	1.221511	1.066832
Average	1.162262	1.003072	1.033933	1.361681	1.216633	1.152925
Std Deviation	0.166856	0.097576	0.082319	0.103713	0.026426	0.130806

ANOVA - IHHNV CHALLENGE ANALYSIS

		Sum of Squares	df	Mean Square	F	Sig.
H0	Between Groups	.039	1	.039	2.599	.182
	Within Groups	.060	4	.015		
	Total	.099	5			
H3	Between Groups	5.660	1	5.660	562.548	.000
	Within Groups	.040	4	.010		
	Total	5.701	5			
H6	Between Groups	44.861	1	44.861	1316.499	.000
	Within Groups	.136	4	.034		
	Total	44.997	5			
H12	Between Groups	4.110	1	4.110	50.162	.002
	Within Groups	.328	4	.082		
	Total	4.438	5			
H24	Between Groups	3.043	1	3.043	556.529	.000
	Within Groups	.022	4	.005		
	Total	3.065	5			
H48	Between Groups	.286	1	.286	8.306	.045
	Within Groups	.138	4	.034		
	Total	.423	5			

APPENDIX G: qRT-PCR ($2^{-\Delta\Delta CT}$) for dsRNA Challenge *Mr*MSTN Gene Expression And One-way ANOVA

	Day 7			Day 14		
	ds-*Mr*MSTN	ds-GFP	CONTROL	ds-*Mr*MSTN	ds-GFP	CONTROL
*Mr*MSTN	28.473	29.382	28.339	29.71	25.65	28.142
	28.519	29.621	28.493	29.3	25.171	28.042
	29.244	29.547	28.933	29.798	25.42	28.396
	29.054	29.605	28.374	29.496	25.31	28.243
	28.8225	29.53875	28.53475	29.576	25.38775	28.20575
EF	25.932	25.964	24.837	27.399	22.044	24.837
	26.281	25.714	24.96	27.277	22.041	24.96
	26.338	25.61	24.785	27.508	22.066	24.785
	26.481	25.662	24.679	27.579	22.008	24.683
	2.8905	3.57475	3.69775	2.177	3.34375	3.36875
	2.5415	3.82475	3.57475	2.299	3.34675	3.24575
	2.4845	3.92875	3.74975	2.068	3.32175	3.42075
	2.3415	3.87675	3.85575	1.997	3.37975	3.52275
	2.5645	3.80125	3.7195	2.13525	3.348	3.3895
	2.5645	2.5645	2.5645	2.13525	2.13525	2.13525
	-0.326	-1.01025	-1.13325	-0.04175	-1.2085	-1.2335
	0.023	-1.26025	-1.01025	-0.16375	-1.2115	-1.1105
	0.08	-1.36425	-1.18525	0.06725	-1.1865	-1.2855
	0.223	-1.31225	-1.29125	0.13825	-1.2445	-1.3875
	1.25353302	2.01426	2.1935233	1.029361693	2.310972	2.3513674
	0.984184022	2.395372	2.0142601	1.120195082	2.315783	2.1592047
	0.946057647	2.574425	2.274028	0.954455605	2.275999	2.4376652
	0.856781955	2.483285	2.4474001	0.908620651	2.369364	2.6162493
Average	**1.010139161**	**2.366836**	**2.2323029**	**1.003158258**	**2.31803**	**2.3911216**
Std Deviation	**0.170820679**	**0.246155**	**0.1798651**	**0.092544666**	**0.038543**	**0.1899251**

ANOVA - dsRNA challenged MrMSTN

		Sum of Squares	df	Mean Square	F	Sig.
DAY7	Between Groups	4.470	2	2.235	54.902	.000
	Within Groups	.366	9	.041		
	Total	4.836	11			
DAY14	Between Groups	4.881	2	2.440	158.740	.000
	Within Groups	.138	9	.015		
	Total	5.019	11			

DAY7

	TEST	N	Subset for alpha = .05	
			1	2
Tukey HSD[a]	1.00	4	1.010139	
	3.00	4		2.232303
	2.00	4		2.366836
	Sig.		1.000	.628
Duncan[a]	1.00	4	1.010139	
	3.00	4		2.232303
	2.00	4		2.366836
	Sig.		1.000	.370

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

DAY14

	TEST	N	Subset for alpha = .05	
			1	2
Tukey HSD[a]	1.00	4	1.003158	
	2.00	4		2.318030
	3.00	4		2.391122
	Sig.		1.000	.693
Duncan[a]	1.00	4	1.003158	
	2.00	4		2.318030
	3.00	4		2.391122
	Sig.		1.000	.426

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

APPENDIX H: Density Cell Calculation

$$\text{Density} = \frac{Nuclei\ Cell}{Size\ (\mu m^2)}$$

Data:

DAY 7									
	MYO			GFP - Positive Control			Negative Control		
Image	S/C	Size (um2)	density (c/um2)	S/C	Size (um2)	density (c/um2)	S/C	Size (um2)	density (c/um2)
1	37	80	0.4625	21	80	0.2625	24	80	0.3
2	35	80	0.4375	19	80	0.2375	27	80	0.3375
3	30	80	0.375	23	80	0.2875	19	80	0.2375
4	31	80	0.3875	28	80	0.35	21	80	0.2625
5	30	80	0.375	28	80	0.35	23	80	0.2875
6	28	80	0.35	23	80	0.2875	21	80	0.2625
7	30	80	0.375	25	80	0.3125	28	80	0.35
8	35	80	0.4375	27	80	0.3375	23	80	0.2875
9	32	80	0.4	30	80	0.375	21	80	0.2625
10	33	80	0.4125	23	80	0.2875	21	80	0.2625
11	36	80	0.45	21	80	0.2625	26	80	0.325
12	31	80	0.3875	26	80	0.325	20	80	0.25
13	37	80	0.4625	20	80	0.25	23	80	0.2875
14	31	80	0.3875	22	80	0.275	22	80	0.275
15	33	80	0.4125	23	80	0.2875	20	80	0.25
16	35	80	0.4375	25	80	0.3125	27	80	0.3375
17	31	80	0.3875	21	80	0.2625	28	80	0.35
18	31	80	0.3875	24	80	0.3	26	80	0.325
19	36	80	0.45	22	80	0.275	25	80	0.3125
20	34	80	0.425	26	80	0.325	21	80	0.2625

ANOVA- Density Calculation

		Sum of Squares	df	Mean Square	F	Sig.
DAY7	Between Groups	.178	2	.089	70.496	.000
	Within Groups	.072	57	.001		
	Total	.250	59			
DAY14	Between Groups	.417	2	.208	76.482	.000
	Within Groups	.155	57	.003		
	Total	.572	59			

DAY7

Tukey HSD [a]

TEST	N	Subset for alpha = .05	
		1	2
3.00	20	.291250	
2.00	20	.298125	
1.00	20		.410000
Sig.		.814	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 20.000.

DAY14

Tukey HSD[a]

TEST	N	Subset for alpha = .05		
		1	2	3
3.00	20	.364375		
2.00	20		.443125	
1.00	20			.566875
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 20.000.

APPENDIX I: Sequence of Selected Growth Regulating Genes

Myosin Heavy Chain

GCGGGAGGGAATTGAATGGACCTTTATTGACTTTGGTCTTGACCTCCAAGCTTGTATT
GACCTCATAGAGAAGCCTCTGGGTATCCTCTCCATCCTAGAAGAGGAGTCTATGTTCC
CTAAGGCTACCGACAAGTCCTTCGAGGAGAAGTTGAAGACCAACCACTTGGGCAAGTC
GCCTAACTTCATCAAGCCCAAGCCACCCAAGCCAGGCCAGGCTGAGGCTCACTTTGCT
ATCGTTCACTACGCCGGCACTGTTCCTTACAACCTCACCGGATGGCTCGAAAAGAACA
AGGATCCCCTGAACGACACCGTCGTGGACCAGGTCAAGAAGGCCTCCAACACCCTGGC
CGTCGAAATCTTCGCCGATCACCCCGGACAGTCGGGCGGTGACCCACAAGCTGGCGGC
AAGGGTGGAAAGCGAGCTAAAGGTTCGGGCTTCTTGACAGTATCCGGGCTTTACAGGG
AACAGCTAAACAACCTGATGACGGTGCTGAGAAGTACAGCTCCTCATTTCATCCGATG
TATCATCCCCAATGAAATAAAGGCGGCTGGTGTGATTGATGCAGCTCTTGTCATGCAT
CAACTGACTTGTAACGGTGTGCTTGAAGGCATCCGTATCTGTCGTAAAGGATTCCCTA
ACAGGATGGTCTACCCTGATTTCAAGCATAGATACGGCATCCTAGCCTCTAAAGCAGC
TAACGCAGCCGAGGATGAAAAGAAAGCAAGTGCTGTCATCCTTGAAGCCATCAACCTG
GAGGCCGAGAAATACAGGATGGGGCATACAAAGGTATTCTTCCGCGCCGGTGTGGTGG
GTACCTTAGAAGAACTTCGAGATGACCGCTTGGCAAAGATTATCTCTTGGATGCAAGC
TTGGATCCGTGGTTACATCAGCCGAAAGACTTACAAGAGACTGCAGGAGCAGCGCGTC
GCCCTTATCGTCGTCCAGCGTAACCTCAGGAAGTTCATGCAACTCCGCACCTGGGCCT
GGTACCGTCTCTGGCAGAAGGTCAAGCCCCTCCTCAACGTCACCCGAGTCGAGGATGA
GATTCGCGCTCTCGAGGAGAAAGCCGCCAAGGCCGAAGAGAACTACGAAAGGGAGGCT
AAGCTGCGAAAGGAGCTCGAGGCCAAGAACCTCGCTCTCCTCGAAGAGAAGAACAACC
TCATGGTGGCTCTCGAATCCACTAAAGGCAATGTAAGCGAGTACCTAGACAAGCAGGC
CAAGTTGCAGAGCCAGAAGGCGGACCTGGAAGCTCAGCTCAATGAAACCACTGAGCGC
CTACAGCAGGAGGAGGAAGCTCGTAATCAGCTCTTCCAAGGCAAGAAGAAGCAGGAAC
AGGAAATCAGCGGACTCAAGAAGGACATTGAAGATCTTGAACTCTCTATACAAAAGGC
TGAACAGGACAAAGCAACTAAAGATCACCAAATTCGCAACCTAAATGATGAGTTCGTC
CTGATGGGCAATCTCA

Tropomyosin

ATGGACGCCATCAAGAAGAAGATGCAGGCGATGAAGCTCGAGAAGGACAACGCCATGG
ACAGGGCGGATACTCTCGAGCAGCAGAACAAGGAGGCCAACAACAGGGCTGAGAAGAC
TGAGGAGGAGATTCGATCGACGCAGAAGAAGATGCAGCAGATCGAGAATGAGCTCGAT
CAGGTTCAGGAGCAGCTCTCCCTTGCTAATACCAAGCTTGAGGAGAAAGAAAAGGCTT
TGCAGAATGCTGAGGGTGAGGTCGCCGCTCTCAACCGCCGCATCCAGCTCCTTGAGGA
AGACCTCGAGAGGTCTGAGGAACGACTCAACACCGCCACCACCAAGTTGGCTGAGGCC
TCCCAGGCAGCCGACGAGTCCGAGCGAGCGCGCAAATGTCTGGAAAACCGAGCAAACA
TGGAAGACGACCGGGTGGGCATCCTGGAGGCTCAACTAGCACAAGCCAAGCACATTGC
CGAGGAGGCCGATAAGAAATATGAAGAGGTTGCCCGTAAACTGGCCATGGTTGAAGCT
GATCTTGAGAGAGCTGAGGAACGCGCAGAAACTGGTGAATCAAAGATCGTCGAGCTTG
AGGAAGAATTGCGCGTCGTTGGCAACAACTTGAAGTCTCTTGAGGTGTCTGAAGAGAA
GTCAGCCCAAAGGGAGGACAAGTTGGAACTCCAGATGAGAAGCTATGCGCAGAGTTTG
AGAGAGGCTGAGGCCCGCGCTGAGTTCGCAGAGAGATCTGTGCAGAAGCTCCAGAAGG
AGGTCGACAGGCTCGAAGACGATCTAGTAGCCGAGAAGGACAAAAATAGAGTGTTGCA
GGCAGATATGGAAGCCACTCTACAGGACATCCAGAATATGTAG

Dystrophin

ATGAGAGTTCTTTCTTTCAAGATCGGTCTCGTCTGCCTTTGCTGCGGTCATTTAGAAG
AAAAATACAGATACATGTTCCGGCTAATTGCCGACCCAAATCGCCTAGTTGATCAAAG
AAAATTAGGGTTATGTTACACGACTGTGTACAAGTTCCACGCCAGTTGGGTGAGGTTG
CAGCATTTGGTGGATCAAACATTGAACCATCAGTTCGTAGCTGTTTCACAAAAGCTGG
AAAGGACAGAGAAACTATTGAGGCT

Farnesoic Acid O-Methyltransferase

ATGGGTGATGACAACTGGCCAGCCTACGGTACCGACGAAAACAAGGAATATAGATTCA
GGCCCATCAAAGGAAAGACCTTGAGATTCCAGGTCAAAGCCGCCCACGATGCTCACGT
TGCCCTTACATCTGGTGCTGAAGAGACTGACCCTATGGTGGAGGTTTTCATTGGAGGA
TGGGAAGGTGCCGCATCAGCTGTTCGCTTCAAGAAAGCTGACGACCTGGTGAAAGTGG
ACACACCCGACATCTTGAGCGAAGAGGAGTACCGAGAGTTCTGGATTGCCTTCGATCA
CGATGAGATCCGAGTGGGCAAGGGGGGAGAGTGGGAGCCTTTCATGATGTGCCCCGTT
CCAGAACCTTTCGGGATCACCCACTACGGTTACTCCACTGGGTGGGGCGCCATCGGCT
GGTGGCAGTTCCACAATGAACGCAAATTCAACAGCGATGACAGCTTGACCTACAACTT
CGAGCCCGCCTACGGCGACACCTTCCACTTCAGCGTCGCCTGCAGCAATGACGCCCAC
TTAGCTTTAACGTCAGGCGCTGAGGAAACCACACCGATGTACGAGATCTTCATCGGTG
GCTGGGATAACCAGAAGTCTGCCATTCGGCTTAGTAAAGGTGAAGATATGATTAAGGT
CGACACCCCTGATGCAGTGTGTTGTGACGAGGAAAGGAAGTTCTACGTCACCTTCAGG
AACGGACACATAAGGGTTGGCTACCAGGACTCTGATCCATTCATGGAGTGGACTGACC
CTGAGCCATGGAAGGTAACTCACGTTGGCTTCTGCACGGGGTGGGGTGCCAGCGGCAA
GTGGCAGTTTGAGATCTAA

Arginine Kinase

ATGGGCGAACCATTCCCTGATATCAAGAGCAAGCACTCCCTGGTGGCCAAGCACGTCA
CCAAGGAGCGCTGGGAGAAACTCTCCGGCCACAAGACCGCCACCTCCGGCTTCACCCT
CAAGCAGGCCATTGCCTGCGCCGTCGAGTTCGACAACCAGCACTGCGGCATCTACGCC
GGTGACTGGGATTCCTACAAGGATTTCAAGGATGTGTTCGACCCCATCATCCAGGAAT
ACCATGGCATCTCCCCCGATGCCGTCCACACCTCCGACATGGAAGTCGAGAAGATCAA
GGGCAACATCAACGCCGAAGTTCCCGTCCACTCCGTCAGGATCCGCGTCGGCCGTAGC
ATCGATGGCTTCGGTCTTTCCCCAGGCATCACCAAGGAACAGCGCATTGGCGTTGAGA
ACCTCATGAAGAGCGCCTTCGGCAAGCTCTCCGGAGATCTCGCCGGCAACTACTACCC
CCTGACTGGCATGGACGAGAAGGTCCGCCAGCAGCTCGTCGATGACCACTTCCTCTTC
ATGTCTGGTGATCGCAACCTTCAGGTCGCAGGTATGGAGCGCGACTGGCCTGAAGGCC
GCGGTATCTACCACAATGCCGAGAAGACCTTCTTGGTCTGGGTCAACGAGGAGGATCA
GCTCAGGATCATCTCCATGCAGATGGGCGGTGATGTCAGGGGCGTCTTCGAGCGCCTG
GCCCGCGGTATCAAGGCCGTCGGTGACTCCGTCAAGGCCGAGAGCGGCAAGGACTTCA
TGCTCGACCCCAAGTACGGCTTCGTCCACTCCTGCCCCACCAACCTTGGCACCGGCAT
GAGGGCTTCCGTCCACGTTGACTTGCCCGGCTGGACCAAGGAAGGTCTTGACGCCCTG
AAGAAGAGATGTGAAGAACTGAAGGTTCAGCCCCGTGGTACCCGCGGTGAATCCGGCG
GCCAGACTGGTCACACTTATGACATCTCCAACAAGCATCGCCTGGGTTACTCCGAGGT
CGAACTCGTCCAGTGCATGATTGACGGTGTCAATACCCTGTACGCTGAGGACGTTGCC
CTCCAGAAGAAGCACGGCATC

Cyclophilin

GAAGCTCCGCGAAAGCCTTCTGCCTTTCAAACTAAGCAGGAGAAGAATTTTCGAGTTC
CTTCTGATACTGCACCAGCGCAGACGGCGGAACGACGACGCCGCATCGGCTCACCATC
ACCGACGTCGCAGGGGAGCACTCAACGGCTCTCTGAACGACGTCCAGCCCCGCCGAAA
CATCCCCGAAGGGACAGTAACAAGACGCAGTGTGATCTACCTTCGTGTAGATGCTGAA
TAAAGCGTCCATCTCGCCCTTCCCGCCGGACGTCCCGACAACTCGCCCTTCGGCGGAC
TTCCCGGCAAATTTACCGTCCCATTCCAGATTTCCCATGAAGTGGCCTCCCGCGTAGG
ACGGGCCCAGAGACCCGAGGCAGAGAGCGAGGAAGTTGTGCGCTCTTCGCAAGTGGCC
CCAGAGGCGTATGTACACCTTCCCGAGGTAGCAGTCGCCGTATGTGACATTTTCGTTC
TCTGCGCTGTTGTCATCGTCACCTGCTGCAGCCAGGAGCTGGAATTCATCTTGCCCCA
TCATACTGGAGACAACTGACTGGATGCTATTCAGCTTGGCCTGACACAGCTCTGCCAG
CTCGACGTCCGTCACTTCACTTGCTTCTTTGCATAAACCAGCGACCTTTACCTTGATG
CAGCTGCGGCAAAACGTGTGGCCACACTGCAGTATCAGGGGATCGTGCTTGCCAGACC
TGTACTCCTCCGAGCACACGGCACAAGTGACGACATTCGTGTGATACAAGGACATCGA
ATGGCATTGAATCCAATCAAGCTCCGCTAGATTTGTTAAACCCCTTCGCAGCTCCACG
AATGGGTCTAATTGAAGCCCCCGCTCCGCTGCACGTATTAGTCTAACGGTCTAACTTC
GTTCACTGCGCAGAATTCTGTCTACTCGATTCCAACGGGAAAAGTCCGAAGGAGACAG
ACGTGCATGACAGACATCTTCGTAACCAGAAGAAACACTGTGCGAAAGAGCCT

Acyl CoA Delta-9 Desaturase

TCATTGATCTAGTGCGCTTACTCTACGCGATCTTGTGCCAAGATATATATTACATATT
TCTGGTGGTGTGACATTAGAATTTTGCTTTACCGCTATGGCGCCACAAGCCCCAGATT
CAGCTGTGAAACATGAAATACTAGATGACCTCACTCCGCAGGAGCATGCTGAGCTTGT
GGAAAGAGAGTTGAAAATTAACCTACGGGAAATCATCTCCAACCTGCGTAATCTGGAC
TGGTCGAAAGTTGTATGGAGAAATCTCATATTATTTGCATTGCTACACATATATTCTG
TTCATGCAACGTGGAAAACCATTTTCTTCAGTGTTGTTTTGTATTTCATGGCCGCCCT
TGGCATTACCATGGGAGCCCATCGCCTTTGGTCACACCGTTCATTCAAGGCGCGCGCA
CCCCTTCGCGTTGTTTTAGCCATCTTTCATAAGTACAGCGAAACAGACGCTGACCCCC
ACAACGCTCGACGAGGATTCTTCTTCTCGCATATGGGATGGCTCATGTACCGTAAACA
CCCAGACGTCATTGCCAAGGGCAAGGGCCTGGACCTCTCCGATCTAGAAAGCGATCCC
ATCGTCATGCTACAACGAAAGTACTACCTGCCTGCTGTCGTCACTGCTTGCTTTTTGT
TGCCAACGGTCATCCCCTGGATGTACTGGGGTGAGCATGTTGTGACTGCTCTCATGGT
CGCCGGTTTTCTGAGGTATACGATAGTATTGCACATCACCTGGTTTGTGAATTCCTTG
GCTTAAAGCCATGAATGATTACTCACCCTTGCAATGCACGTCACGGAACAATAAGCGC
ACACAGGCCTTGCTCTTTGGGTACAGCATCCAATCCCTCTTCCCCT

APPENDIX J: qRT-PCR ($2^{-\Delta\Delta CT}$) for Myosin Heavy Chain and One-way ANOVA

	Day 7			Day 14		
	ds-*Mr* MSTN	ds-GFP	CONTROL	ds-*Mr* MSTN	ds-GFP	CONTROL
MhC	28.395	28.649	27.18	28.523	27.153	27.278
	27.81	28.238	27.445	28.575	27.015	27.133
	27.813	28.306	27.168	28.536	27.338	27.388
	28.157	28.292	27.087	28.452	27.351	27.278
	28.04375	28.37125	27.22	28.5215	27.21425	27.26925
EF	25.962	24.907	23.855	26.932	23.609	23.837
	26.001	24.922	23.94	26.281	23.6	23.96
	25.936	25.054	23.764	26.338	23.535	23.785
	26.338	24.964	23.653	26.425	23.609	23.647
	2.08175	3.46425	3.365	1.5895	3.60525	3.43225
	2.04275	3.44925	3.28	2.2405	3.61425	3.30925
	2.10775	3.31725	3.456	2.1835	3.67925	3.48425
	1.70575	3.40725	3.567	2.0965	3.60525	3.62225
	1.9845	3.4095	3.417	2.0275	3.626	3.462
	3.417	3.417	3.417	3.462	3.462	3.462
	1.33525	-0.04725	0.052	1.8725	-0.14325	0.02975
	1.37425	-0.03225	0.137	1.2215	-0.15225	0.15275
	1.30925	0.09975	-0.039	1.2785	-0.21725	-0.02225
	1.71125	0.00975	-0.15	1.3655	-0.14325	-0.16025
	0.39632338	1.033293	0.9645982	0.273099769	1.10439	0.97959
	0.38575319	1.022606	0.9094083	0.428836616	1.111301	0.899534
	0.4035306	0.933195	1.0274014	0.412223884	1.162516	1.015542
	0.30539535	0.993265	1.1095695	0.388099909	1.10439	1.117481
Average	**0.37275063**	**0.99559**	**1.0027443**	**0.375565044**	**1.120649**	**1.003037**
Std.Deviation	**0.04549316**	**0.044907**	**0.0859968**	**0.070327771**	**0.0281**	**0.0904**

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
DAY7	Between Groups	1.046	2	.523	136.717	.000
	Within Groups	.034	9	.004		
	Total	1.081	11			
DAY14	Between Groups	1.284	2	.642	138.441	.000
	Within Groups	.042	9	.005		
	Total	1.325	11			

DAY7

Tukey HSD [a]

		Subset for alpha = .05	
TEST	N	1	2
1.00	4	********	
2.00	4		********
3.00	4		********
Sig.		1.000	.985

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

DAY14

Tukey HSD [a]

		Subset for alpha = .05	
TEST	N	1	2
1.00	4	********	
3.00	4		********
2.00	4		********
Sig.		1.000	.086

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

APPENDIX K: qRT-PCR ($2^{-\Delta\Delta CT}$) for Dystrophin-Dystroglycan and One-way ANOVA

	Day 7			Day 14		
	ds-*Mr*MSTN	ds-GFP	CONTROL	ds-*Mr*MSTN	ds-GFP	CONTROL
Dystrophin	29.251	28.467	29.063	29.527	27.878	29.115
	29.05	28.219	29.011	29.594	27.718	29.001
	29.09	28.055	29.166	29.222	27.651	29.059
	29.201	28.229	29.264	29.54	27.75	29.166
	29.148	28.2425	29.126	29.47075	27.74925	29.08525
EF	25.932	23.907	24.837	26.46366667	23.984	24.627
	26.281	23.922	24.96	26.39466667	23.574	24.96
	26.338	23.954	24.785	26.86466667	23.826	24.785
	25.933	23.907	24.922	26.42366667	23.566	24.922
	3.216	4.3355	4.289	3.007083333	3.76525	4.45825
	2.867	4.3205	4.166	3.076083333	4.17525	4.12525
	2.81	4.2885	4.341	2.606083333	3.92325	4.30025
	3.215	4.3355	4.204	3.047083333	4.18325	4.16325
	3.027	4.32	4.25	2.934083333	4.01175	4.26175
	4.25	4.25	4.25	4.26175	4.26175	4.26175
	1.034	-0.0855	-0.039	1.254666667	0.4965	-0.1965
	1.383	-0.0705	0.084	1.185666667	0.0865	0.1365
	1.44	-0.0385	-0.091	1.655666667	0.3385	-0.0385
	1.035	-0.0855	0.046	1.214666667	0.0785	0.0985
	0.488354264	1.061055	1.027401	0.419090387	0.708824	1.145915
	0.383420665	1.050081	0.943438	0.439621343	0.941805	0.909723
	0.368567304	1.027045	1.065108	0.317391046	0.790863	1.027045
	0.48801588	1.061055	0.968618	0.430872621	0.947042	0.934004
Average	**0.432089529**	**1.049809**	**1.001142**	**0.401743849**	**0.847134**	**1.004172**
Std.Deviation	**0.06505692**	**0.016033**	**0.055283**	**0.056860905**	**0.117246**	**0.107173**

ANOVA- Dystrophin

		Sum of Squares	df	Mean Square	F	Sig.
DAY7	Between Groups	.718	2	.359	25.565	.000
	Within Groups	.126	9	.014		
	Total	.844	11			
DAY14	Between Groups	.636	2	.318	21.323	.000
	Within Groups	.134	9	.015		
	Total	.770	11			

DAY7

Tukey HSD[a]

		Subset for alpha = .05	
DYSTRO	N	1	2
1.00	4	********	
3.00	4		********
2.00	4		********
Sig.		1.000	.834

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

DAY14

Tukey HSD[a]

		Subset for alpha = .05	
DYSTRO	N	1	2
1.00	4	********	
2.00	4		********
3.00	4		********
Sig.		1.000	.463

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

APPENDIX L: qRT-PCR ($2^{-\Delta\Delta CT}$) for Tropomyosin and One-way ANOVA

	Day 7			Day 14		
	ds-*Mr*MSTN	ds-GFP	CONTROL	ds-*Mr*MSTN	ds-GFP	CONTROL
Tropomyosin	31.91566667	29.071	28.354	33.029	26.692	28.354
	31.04566667	29.235	28.841	33.506	26.463	28.841
	31.50533333	29.176	28.037	33.085	26.42	28.037
	31.964	29.218	28.306	33.982	26.306	28.306
	31.60766667	29.175	28.3845	33.4005	26.47025	28.3845
EF	25.932	24.907	23.874	26.425	22.044	23.874
	26.281	24.922	23.929	26.372	22.041	23.929
	26.338	25.054	23.938	26.594	22.066	23.938
	25.923	25.054	23.929	26.508	22.069	23.647
	5.675666667	4.268	4.5105	6.9755	4.42625	4.5105
	5.326666667	4.253	4.4555	7.0285	4.42925	4.4555
	5.269666667	4.121	4.4465	6.8065	4.40425	4.4465
	5.684666667	4.121	4.4555	6.8925	4.40125	4.7375
	5.489166667	4.19075	4.467	6.92575	4.41525	4.5375
	4.467	4.467	4.467	4.5375	4.5375	4.5375
	-1.208666667	0.199	-0.0435	-2.438	0.11125	0.027
	-0.859666667	0.214	0.0115	-2.491	0.10825	0.082
	-0.802666667	0.346	0.0205	-2.269	0.13325	0.091
	-1.217666667	0.346	0.0115	-2.355	0.13625	-0.2
	2.311239342	0.8711542	1.030611074	5.41889991	0.925786	0.98145906
	1.814618996	0.8621435	0.992060493	5.621674801	0.927713	0.94474704
	1.744322341	0.7867624	0.985890962	4.81988926	0.911775	0.93887175
	2.32570267	0.7867624	0.992060493	5.115942325	0.909881	1.14869835
Average	**2.048970837**	**0.8267057**	**1.000155756**	**5.244101574**	**0.918789**	**1.00344405**
Std.Deviation	**0.312568268**	**0.0462689**	**0.020510789**	**0.350949607**	**0.009258**	**0.09865271**

ANOVA-TROPOMYOSIN

		Sum of Squares	df	Mean Square	F	Sig.
DAY7	Between Groups	3.499	2	1.749	52.344	.000
	Within Groups	.301	9	.033		
	Total	3.799	11			
DAY14	Between Groups	48.932	2	24.466	551.927	.000
	Within Groups	.399	9	.044		
	Total	49.331	11			

DAY7

Tukey HSD[a]

		Subset for alpha = .05	
TEST	N	1	2
2.00	4	********	
3.00	4	********	
1.00	4		********
Sig.		.409	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

DAY14

Tukey HSD[a]

		Subset for alpha = .05	
TEST	N	1	2
2.00	4	********	
3.00	4	********	
1.00	4		********
Sig.		.840	1.000

Means for groups in homogeneous subsets are displaye

a. Uses Harmonic Mean Sample Size = 4.000.

APPENDIX M: qRT-PCR ($2^{-\Delta\Delta CT}$) for Farnesoic Acid O-Methyl Transferase and One-way ANOVA

	Day 7			Day 14		
	ds-*Mr*MSTN	ds-GFP	CONTROL	ds-*Mr*MSTN	ds-GFP	CONTROL
FaMET	24.101	21.969	21.37	27.667	23.633	23.837
	24.145	22.033	21.487	28.215	23.711	23.874
	24.283	21.828	21.337	27.826	23.841	23.929
	24.316	21.928	21.376	27.682	23.657	23.938
	24.21125	21.9395	21.3925	27.8475	23.7105	23.8945
EF	25.932	24.907	24.837	26.425	23.044	23.37
	26.281	24.922	24.96	26.372	23.041	23.487
	26.338	25.054	24.785	26.594	23.066	23.337
	25.962	24.954	24.922	26.542	23.036	23.37
	-1.72075	-2.9675	-3.4445	1.4225	0.6665	0.5245
	-2.06975	-2.9825	-3.5675	1.4755	0.6695	0.4075
	-2.12675	-3.1145	-3.3925	1.2535	0.6445	0.5575
	-1.75075	-3.0145	-3.5295	1.3055	0.6745	0.5245
	-1.917	-3.01975	-3.4835	1.36425	0.66375	0.5035
	-3.4835	-3.4835	-3.4835	0.5035	0.5035	0.5035
	-1.76275	-0.516	-0.039	-0.919	-0.163	-0.021
	-1.41375	-0.501	0.084	-0.972	-0.166	0.096
	-1.35675	-0.369	-0.091	-0.75	-0.141	-0.054
	-1.73275	-0.469	0.046	-0.802	-0.171	-0.021
	3.393443517	1.429985	1.02740144	1.890804234	1.119613	1.014663
	2.664287924	1.4151942	0.94343825	1.961558008	1.121943	0.935623
	2.561075884	1.2914574	1.0651082	1.681792831	1.102669	1.038139
	3.323607459	1.3841497	0.96861819	1.743516479	1.125839	1.014663
Average	**2.985603696**	**1.3801966**	**1.00114152**	**1.819417888**	**1.117516**	**1.000772**
Std.Deviation	**0.433607946**	**0.0621664**	**0.05528337**	**0.129101387**	**0.010226**	**0.04482**

ANOVA-FaMET

		Sum of Squares	df	Mean Square	F	Sig.
DAY7	Between Groups	8.879	2	4.439	68.321	.000
	Within Groups	.585	9	.065		
	Total	9.464	11			
DAY14	Between Groups	1.001	2	.501	10.169	.005
	Within Groups	.443	9	.049		
	Total	1.444	11			

DAY7

Tukey HSD[a]

		Subset for alpha = .05	
FAMET	N	1	2
3.00	4	********	
2.00	4	********	
1.00	4		********
Sig.		.144	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

DAY14

Tukey HSD[a]

		Subset for alpha = .05	
FAMET	N	1	2
3.00	4	********	
2.00	4	********	
1.00	4		********
Sig.		.745	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

APPENDIX N: qRT-PCR ($2^{-\Delta\Delta CT}$) for Arginine Kinase and One-way ANOVA

	Day 7			Day 14		
	ds-*Mr*MSTN	ds-GFP	CONTROL	ds-*Mr*MSTN	ds-GFP	CONTROL
AgK	25.646	25.026	24.262	27.366	23.072	23.602
	25.609	25.092	24.38	27.328	23.146	23.446
	25.391	24.833	24.559	27.58	23.061	23.357
	25.463	24.796	24.365	27.491	23.091	23.561
	25.52725	24.93675	24.3915	27.44125	23.0925	23.4915
EF	25.962	24.907	24.837	26.425	23.044	23.37
	26.001	24.922	24.96	26.372	23.041	23.487
	25.936	25.054	24.785	26.594	23.066	23.337
	25.932	25.099	24.922	26.534	23.039	23.37
	-0.43475	0.02975	-0.4455	1.01625	0.0485	0.1215
	-0.47375	0.01475	-0.5685	1.06925	0.0515	0.0045
	-0.40875	-0.11725	-0.3935	0.84725	0.0265	0.1545
	-0.40475	-0.16225	-0.5305	0.90725	0.0535	0.1215
	-0.4305	-0.05875	-0.4845	0.96	0.045	0.1005
	-0.4845	-0.4845	-0.4845	0.1005	0.1005	0.1005
	-0.04975	-0.51425	-0.039	-0.91575	0.052	-0.021
	-0.01075	-0.49925	0.084	-0.96875	0.049	0.096
	-0.07575	-0.36725	-0.091	-0.74675	0.074	-0.054
	-0.07975	-0.32225	0.046	-0.80675	0.047	-0.021
	1.035085542	1.42825146	1.027401439	1.88654956	0.9645982	1.0146625
	1.007479162	1.41347856	0.943438251	1.95714412	0.9666061	0.9356235
	1.053908779	1.28989175	1.065108203	1.67800847	0.9500004	1.0381393
	1.056834889	1.25027894	0.968618189	1.74926638	0.967947	1.0146625
Average	**1.038327093**	**1.34547518**	**1.001141521**	**1.81774213**	**0.9622879**	**1.000772**
Std.Deviation	**0.02271145**	**0.08874723**	**0.055283374**	**0.12699318**	**0.0083065**	**0.0448201**

ANOVA- Arginine Kinase

		Sum of Squares	df	Mean Square	F	Sig.
DAY7	Between Groups	.226	2	.113	6.972	.015
	Within Groups	.146	9	.016		
	Total	.371	11			
DAY14	Between Groups	1.868	2	.934	153.882	.000
	Within Groups	.055	9	.006		
	Total	1.922	11			

DAY7

Tukey HSD[a]

		Subset for alpha = .05	
AK	N	1	2
3.00	4	********	
1.00	4	********	********
2.00	4		********
Sig.		.360	.114

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

DAY14

Tukey HSD[a]

		Subset for alpha = .05	
AK	N	1	2
2.00	4	********	
3.00	4	********	
1.00	4		********
Sig.		.770	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

APPENDIX O: qRT-PCR ($2^{-\Delta\Delta CT}$) for Cyclophilin and One-way ANOVA

	Day 7			Day 14		
	ds-*Mr*MSTN	ds-GFP	CONTROL	ds-*Mr*MSTN	ds-GFP	CONTROL
Cyclophilin	27.951	28.219	28.252	28.564	28.442	28.804
	28.322	28.237	28.572	28.692	28.581	28.659
	28.338	28.043	28.147	28.858	28.515	28.843
	27.619	28.527	28.251	28.568	28.591	28.882
	28.0575	28.2565	28.3055	28.6705	28.53225	28.797
EF	25.932	24.907	24.837	26.425	24.609	24.943
	26.281	24.922	24.96	26.372	24.6	24.857
	26.338	24.907	24.785	26.594	24.535	25.029
	25.962	24.922	24.786	26.435	24.566	25.029
	2.1255	3.3495	3.4685	2.2455	3.92325	3.854
	1.7765	3.3345	3.3455	2.2985	3.93225	3.94
	1.7195	3.3495	3.5205	2.0765	3.99725	3.768
	2.0955	3.3345	3.5195	2.2355	3.96625	3.768
	1.92925	3.342	3.4635	2.214	3.95475	3.8325
	3.4635	3.4635	3.4635	3.8325	3.8325	3.8325
	1.338	0.114	-0.005	1.587	-0.09075	-0.0215
	1.687	0.129	0.118	1.534	-0.09975	-0.1075
	1.744	0.114	-0.057	1.756	-0.16475	0.0645
	1.368	0.129	-0.056	1.597	-0.13375	0.0645
	0.39556865	0.924023	1.003472	0.332862903	1.064924	1.015014
	0.31057207	0.914465	0.921464	0.345318612	1.071588	1.07736
	0.2985408	0.924023	1.0403	0.296067903	1.120972	0.956277
	0.38742797	0.914465	1.039579	0.330563651	1.097142	0.956277
Average	**0.34802737**	**0.919244**	**1.001204**	**0.326203267**	**1.088656**	**1.001232**
Std.Deviation	**0.05054503**	**0.005518**	**0.055871**	**0.021110031**	**0.025631**	**0.057814**

ANOVA- Cyclophilin

		Sum of Squares	df	Mean Square	F	Sig.
DAY7	Between Groups	1.013	2	.506	266.223	.000
	Within Groups	.017	9	.002		
	Total	1.030	11			
DAY14	Between Groups	1.393	2	.696	470.026	.000
	Within Groups	.013	9	.001		
	Total	1.406	11			

DAY7

Tukey HSD [a]

		Subset for alpha = .05	
CYP	N	1	2
1.00	4	********	
2.00	4		********
3.00	4		********
Sig.		1.000	.062

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

DAY14

Tukey HSD[a]

		Subset for alpha = .05		
CYP	N	1	2	3
1.00	4	********		
3.00	4		********	
2.00	4			********
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

APPENDIX P: qRT-PCR ($2^{-\Delta\Delta CT}$) for Acyl CoA Desaturase and One-way ANOVA

	Day 7			Day 14		
	ds-*Mr* MSTN	ds-GFP	CONTROL	ds-*Mr* MSTN	ds-GFP	CONTROL
Acyl Co A -D	31.123	31.909	32.177	31.862	30.037	32.577
	30.954	31.94	32.187	31.808	30.14	32.187
	30.365	31.096	32.139	31.324	30.321	32.139
	30.754	32.013	32.032	31.921	30.308	32.032
	30.799	31.7395	32.13375	31.72875	30.2015	32.23375
EF	25.932	24.907	24.943	26.765	23.044	24.943
	26.281	24.922	24.857	26.833	23.041	24.857
	26.338	25.054	25.029	26.996	23.066	25.029
	26.238	24.977	25.129	26.875	23.042	24.769
	4.867	6.8325	7.19075	4.96375	7.1575	7.29075
	4.518	6.8175	7.27675	4.89575	7.1605	7.37675
	4.461	6.6855	7.10475	4.73275	7.1355	7.20475
	4.561	6.7625	7.00475	4.85375	7.1595	7.46475
	4.60175	6.7745	7.14425	4.8615	7.15325	7.33425
	7.14425	7.14425	7.14425	7.33425	7.33425	7.33425
	2.27725	0.31175	-0.0465	2.3705	0.17675	0.0435
	2.62625	0.32675	-0.1325	2.4385	0.17375	-0.0425
	2.68325	0.45875	0.0395	2.6015	0.19875	0.1295
	2.58325	0.38175	0.1395	2.4805	0.17475	-0.1305
	0.206290602	0.805664	1.032756	0.193378593	0.884693728	0.97029813
	0.161964552	0.797331	1.096192	0.184475355	0.886535311	1.02989696
	0.155690196	0.727616	0.972992	0.164767088	0.871305165	0.91414821
	0.16686462	0.767506	0.907834	0.179182295	0.885921024	1.09467302
Average	**0.172702492**	**0.774529**	**1.002443**	**0.180450833**	**0.882113807**	**1.00225408**
Std.Deviation	**0.022854346**	**0.035305**	**0.080676**	**0.01198489**	**0.007246317**	**0.07765139**

ANOVA - AcylCoA

		Sum of Squares	df	Mean Square	F	Sig.
DAY7	Between Groups	1.470	2	.735	266.413	.000
	Within Groups	.025	9	.003		
	Total	1.495	11			
DAY14	Between Groups	1.576	2	.788	379.745	.000
	Within Groups	.019	9	.002		
	Total	1.595	11			

DAY7

Tukey HSD[a]

		Subset for alpha = .05		
ACYLCOA	N	1	2	3
1.00	4	********		
2.00	4		********	
3.00	4			********
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

DAY14

Tukey HSD[a]

		Subset for alpha = .05		
ACYLCOA	N	1	2	3
1.00	4	********		
2.00	4		********	
3.00	4			********
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.