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MYOSTATIN AND TGF- β FUNCTION IN THE REGULATION OF MYOGENESIS
IN RAINBOW TROUT (*Oncorhynchus mykiss*), A BASAL TELEOST

BY

IAN MORRISON JAFFE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

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2013

MASTER OF SCIENCE THESIS

OF

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2013

ABSTRACT

Myogenesis in vertebrates is a complex and intricately regulated process, various mechanisms of which have been alternately conserved or lost throughout the course of vertebrate evolution. Mammalian myogenesis is highly regulated by myostatin, a member of the Transforming Growth Factor- β (TGF- β) family of cytokines, however, a similar role for myostatin in fish currently remains the subject of inquiry. Myostatin initiates signal transduction by means of the activin type IIb receptor, and shares considerable sequence and structural homology across vertebrate taxa, though its functional homology remains unclear. Two families of transgenic (TG) rainbow trout (RBT), one in which myostatin was inhibited (PD), and another in which myostatin and related TGF- β ligands were collectively inhibited (ActRIIB), were utilized to investigate the growth, morphology, muscle phenotype, and downstream genetic activity proceeding from myostatin/TGF- β inhibition. Additionally, the well established function of rbST in rendering increased somatogenesis was investigated in these two families; groups of TG and non-transgenic (NTG) fish received either a dosage of rbST ($50 \mu\text{g g}^{-1}$ body weight) or placebo (sesame oil). Growth and muscularity in TG fish were hypothesized to surpass that observed in NTG fish in the context of myostatin inhibition. Administration of rbST resulted in accelerated growth and increased body size in both genotypes in the ActRIIB and PD families. Growth rate for fish in both families did not differ between genotypes for fish receiving identical treatments, suggesting that myostatin/TGF- β inhibition does not augment growth rate in RBT. Neither inhibitory mechanism increased body size in TG fish beyond that of NTG individuals, demonstrating that these effectors do not serve a predominant role in constraining absolute growth. While TG

pro-domain fish maintained body size and conformation equivalent to that of NTG fish within both treatment groups, ActRIIB TG fish remained significantly shorter, with a significantly lesser body mass and higher condition factor than NTG fish, irrespective of treatment. The findings presented here suggest that one or more TGF- β molecules, excluding myostatin, that bind the activin type IIb receptor appear to be involved in axial patterning and growth potential. A role for a TGF- β molecule other than myostatin in restricting the capacity for hyperplasia is supported by the increased myofiber density of the epaxial musculature. Myostatin and related TGF- β molecules were found to impede expression of the myogenic regulatory factor *MyoD*, however, increased expression failed to enhance myogenesis. *Myogenin* expression was not influenced by the reduction in myostatin/TGF- β signaling. These data collectively suggest that myostatin and associated TGF- β molecules are not functioning to restrict muscle growth in basal teleosts as they are in mammals, although some TGF- β ligands may be involved in limiting myogenic proliferation. TGF- β molecules other than myostatin do appear to function to some extent in somite architecture and patterning, and may be necessary to maintain growth potential. Collectively, these findings support the proposal that myostatin has evolved differing capacities within the context of disparate vertebrate physiologies.

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DEDICATION

This thesis is dedicated to my aunt, whose capacity for kindness and love were boundless, and inspiring, and who brought profound happiness to those who had the privilege of having her in their life.

PREFACE

The following thesis is written in manuscript format and adheres to the guidelines established by the Graduate School of the University of Rhode Island. It is comprised of one manuscript, a literature review, and a bibliography; the manuscript is formatted in accordance with the guidelines set forth for publication in the *Journal of Experimental Biology*.

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LIST OF ABBREVIATIONS

ActRIIB: *Danio rerio* transgene construct encoding truncated activin IIb receptor; fish in the family containing this construct

IGF: Insulin-like growth factor

NTG: Non-transgenic fish

PD: Transgene construct encoding amino-terminal domain of the myostatin protein; fish in the family containing this construct

rbST: Recombinant bovine somatotropin

RBT: Rainbow trout (*Oncorhynchus mykiss*)

TG: Transgenic fish

TGF- β : Transforming Growth Factor- β

MANUSCRIPT I

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MYOSTATIN AND TGF- β FUNCTION IN THE REGULATION OF MYOGENESIS IN RAINBOW TROUT (*Oncorhynchus mykiss*), A BASAL TELEOST

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1. INTRODUCTION

The physiology of vertebrate growth is intricately regulated and highly complex, mediated to varying degrees by genetic, epigenetic, endocrine, and environmental factors that alternately promote and restrict somatogenesis. Growth in many teleost species, including salmonids, is unique in that it is indeterminate, persisting throughout ontogeny. The processes of hypertrophy and hyperplasia that occur in the skeletal muscle produce a heterogeneous landscape of fibers within the serially repeating myotomes that comprise the trunk musculature (Johnston et al., 2011; Jobling, 1994; Rescan, 2005). Deciphering the mechanisms regulating skeletal myogenesis in rainbow trout (*Oncorhynchus mykiss*) (RBT) facilitates understanding of a more basal physiology of myogenic regulation and the evolution of a prominent component of somatogenesis.

The necessity for growth physiologies to operate in a manner that remains integrated with the host of other physiological processes taking place within an organism has led to the evolution of a versatile regulation of muscle development. Insulin-like growth factors-I and -II (IGF-I and IGF-II), somatotropin, insulin, and the somatotrophic axis of which these endocrine effectors are a part, have long been understood to be integral to myogenesis (Butler and LeRoith, 2001; Johnston et al., 2010). Myostatin, a member of the transforming growth factor- β (TGF- β) superfamily of endocrine cytokines, has recently been discovered to be a requisite factor in mammalian myogenesis (McPherron and Lee, 1997). The protein is a negative regulator of skeletal muscle, exerting control over cell cycle progression by binding to a transmembrane receptor complex which transduces an inhibitory signal to intracellular effectors (Liu et al., 2001; Thomas et al., 2000). However, the extent to which myostatin is involved in

myogenic regulation in teleosts, and the conservation of its physiological function across vertebrate clades, remains to be conclusively elucidated. In an effort to better resolve the function of myostatin in teleosts, and define potentially conserved functions among vertebrates, somatic growth was induced in myostatin- and myostatin/TGF- β knockdown transgenic rainbow trout by means of exogenous somatotropin (rbST). The utilization of two distinct inhibitory mechanisms, one targeting myostatin as well as related TGF- β s and the other inhibiting myostatin exclusively, enabled the examination of the role of myostatin and related cytokines in teleostean myogenesis.

Myostatin has a crucial function in skeletal myogenesis in mammals (McPherron et al., 1997). It operates as a negative regulator of skeletal muscle, interfering with the processes of myoblast differentiation and proliferation (Thomas et al., 2000; Taylor et al., 2001; Langley et al., 2002; Liu et al., 2001). Non-functional *myostatin* alleles in a number of domesticated mammalian species have produced a conspicuous phenotype characterized by excessive skeletal muscle mass, referred to as double-muscling (McPherron and Lee, 1997; Kambadur et al., 1997; Clop et al., 2006; Mosher et al., 2007). A similar phenotype has been observed in the skeletal musculature of humans with myostatin deficiencies (Schuelke et al., 2004). Distinct mutations, one involving a frameshift subsequent to an oligonucleotide deletion and the other resulting in the loss of a cysteine residue integral to structural conformation in the Belgian Blue and Piedmontese cattle breeds, have produced dramatically increased musculature (20-25 percent) resulting from hypertrophic and hyperplastic increases (McPherron and Lee, 1997; Kambadur et al., 1997).

The conformation of mammalian myostatin is defined by the structural hallmarks characteristic of proteins of the TGF- β superfamily, including a secretory domain, a conserved RXXR sequence directing proteolytic cleavage, and a highly conserved carboxy-terminal domain distinguished by several cysteine residues (McPherron et al., 1997; McPherron and Lee, 1997). These cysteine residues, and their respective positions, are essential for the disulfide bonds that form among them to facilitate formation of the cysteine knot common to TGF- β ligands and requisite for their functionality (McPherron et al., 1997; McPherron and Lee, 1997). The predicted amino acid sequence of myostatin demonstrates considerable conservation of the proteolytic cleavage site, as well as the biologically active domain of the protein. The amino acid sequence of zebrafish (*Danio rerio*) shares substantial sequence homology with that of more derived vertebrates, including murine, avian, and porcine species (Rescan et al., 2001; McPherron and Lee, 1997).

Myostatin is initially translated as a physiologically inactive latent precursor. The biologically active carboxy-terminal domain remains confined within a complex comprised of the amino-terminal pro-domain region of the peptide (additionally referred to as the latency associated protein, LAP) along with other proteins, e.g., the latent TGF- β binding protein (LTBP) (Lee and McPherron, 2001; Munger et al., 1997; Thomas et al., 2000; de Caestecker, 2004). Following secretion, the latent myostatin complex appears to remain bound to the extracellular matrix; proteolytic or non-proteolytic cleavage subsequently liberates myostatin, though the active domain continues to be bound by the amino-terminal pro-domain, rendering it inactive (Lee and McPherron, 2001; Munger et al., 1997). Even as a latent precursor, disulfide bonds appear to facilitate formation of

myostatin homodimers (Lee and McPherron, 2001). The biochemically active, mature carboxy-terminal myostatin dimer is produced only when proteolytic cleavage by BMP-1/metalloproteinase family members separates it from the pro-domain, yielding a physiologically viable cytokine (Lee, 2004; Lee and McPherron, 2001). It is this cytokine that binds the extracellular domain of the Activin type IIB serine-threonine kinase receptor, subsequently recruiting the ALK4 (Activin-like kinase), a type I serine-threonine kinase receptor, thereby forming a heteromeric receptor complex (Lee and McPherron, 2001; Lee, 2004; Kemaladewi et al., 2012; Derynck and Zhang, 2003). Myostatin binding initiates a Smad-mediated signaling cascade that ultimately interferes with myogenic transcription factors such as MyoD and the expression of myogenic genes (Ge et al., 2011; Massagué, 2000; Liu et al., 2001). Disruption of myogenic gene expression, along with the up-regulation of cyclin-dependent kinase (CDK) inhibitors such as p21, which inhibits myoblast proliferation, facilitate the inhibitory role of myostatin (Thomas et al., 2000; Joulia-Ekaza and Cabello, 2006; Langley et al., 2002; Taylor et al., 2001).

Recent data have demonstrated that increases in muscle mass in mammals occur through hypertrophy of the existing tissue and rely minimally upon satellite cell activation and recruitment (Wang and McPherron, 2012; Amthor et al., 2009). These findings suggest that myostatin inhibits protein accretion within the skeletal muscle and myofiber synthesis, rather than inhibiting the proliferation and differentiation of satellite and precursor cell populations (Amthor et al., 2009; Wang and McPherron, 2012). However, these investigations have been conducted in murine models that exhibit determinate growth rather than teleost species possessing indeterminate growth potential.

Myostatin is subject to regulation by disparate mechanisms at the genome level and those acting on the signaling cascades or the circulating ligand. The amino-terminal pro-domain region of the myostatin molecule itself functions as an endogenous regulatory mechanism as the active carboxy-terminal domain remains latent when bound to it, only becoming physiologically viable upon cleavage (Lee and McPherron, 2001). Follistatin binds to, and suppresses, myostatin as well as other TGF- β ligands in a variety of vertebrate taxa (Haidet et al., 2008; Lee et al., 2010; Medeiros et al., 2009; Rebhan and Funkenstein, 2008). Myostatin, in the latent and/or active form(s) may be variously bound in circulation and/or within skeletal muscle by factors including titan-cap, GASP-1 (growth and differentiation factor-associated serum protein-1), FLRG (follistatin-related gene), and hSGT (human small glutamine-rich tetratricopeptide repeat-containing protein), all of which operate as myostatin antagonists (Hill et al., 2003; Hill et al., 2002; Wang et al., 2003; Joulia-Ekaza and Cabello, 2006).

Experimental strategies employed to inhibit myostatin, individually or in conjunction with other TGF- β ligands, have produced phenotypes paralleling those observed in animals possessing wild-type loss-of-function *myostatin* mutations (Lee and McPherron, 2001). Interfering with TGF- β signaling in an experimental context has demonstrated that myostatin is but one of an as yet unknown number of regulatory factors involved in restraining myogenesis, among them Activin A (Lee et al., 2005; Lee et al., 2010; Lee, 2007). The involvement of additional negative regulators has been demonstrated by the increased muscling observed in *myostatin* null mice simultaneously overexpressing follistatin (Lee, 2007) or a soluble Activin IIB receptor (Lee et al., 2005), which antagonize multiple TGF- β ligands concomitant with myostatin. *Myostatin* null

mice displayed a double-muscle phenotype resembling that observed in cattle breeds with myostatin deficiencies, as well as significantly increased skeletal muscle mass (Lee, 2007; Lee and McPherron, 2001; McPherron et al. 1997). Hypertrophic and hyperplastic growth were both found to contribute to the superior musculature of transgenic mice expressing either follistatin or a dominant negative isoform of the Activin IIB receptor (Lee and McPherron, 2001). Transgenic expression of follistatin or the pro-domain region of the endogenous myostatin peptide similarly enhanced muscle hypertrophy (Lee et al., 2010; Lee, 2007; Yang et al., 2001; Thies et al., 2001).

In teleosts, the function of myostatin, as well as those of many related TGF- β proteins, remains unresolved, owing perhaps to factors such as diverse growth modes, genome duplication events, and unique muscle physiologies. Myostatin is nearly ubiquitously expressed in the tissues of bony fish (Radaelli et al., 2003), the paralogs of which exhibit tissue-specific differential expression, particularly in the muscle (Roberts and Goetz, 2001; Østbye et al., 2007; Rescan et al., 2001). Overexpression of the endogenous myostatin pro-domain in transgenic zebrafish resulted in spatially discrete, that is, stratified rather than mosaic, hyperplasia of the skeletal muscle; the extent of the hyperplasia, however, was minimal and hypertrophy was not induced (Xu et al., 2003). Binding of gilthead seabream (*Sparus auratus*) myostatin by both follistatin and pro-domain constructs has been demonstrated *in vitro* (Rebhan and Funkenstein, 2008). Previous work in our lab demonstrated that transgenic rainbow trout (*Oncorhynchus mykiss*) overexpressing a follistatin construct exhibited a uniquely muscular phenotype in the lateral muscle, and hyperplasia of the epaxial and hypaxial skeletal muscle tissue (Medeiros et al., 2009).

The functionality of the somatotrophic axis, and endocrine factors including IGF-I and somatotropin (Stitt et al., 2004; Cleveland and Weber, 2009; Seiliez et al., 2010), in vertebrate myogenesis have been better defined than that of myostatin, and may represent an opportunity for improved understanding of the role of myostatin in muscle development. The somatotrophic axis and growth physiology have been comprehensively investigated in teleosts, in particular salmonids, not merely to provide context for the evolution of somatogenic mechanisms, many of which are homologous in vertebrates, including rainbow trout (Björnsson et al., 2002; Greene and Chen, 1999; Wood et al., 2005; LeRoith et al., 2001), but because exploiting these pathways has the potential to enhance commercial aquaculture (Levesque et al., 2008; de-Santis and Jerry, 2007). Somatic indices in salmonids have been augmented through both the introduction of exogenous growth hormone (Garber et al., 1995) and incorporation of growth hormone transgene constructs (Levesque et al., 2008). Myogenic regulatory factors, as well as molecular effectors along the somatotrophic axis, exhibited similar responses in their transcriptional profiles to the anabolic effects of exogenous hormone administration and genetic manipulations (Devlin et al., 2009; Biga et al., 2005; Biga et al., 2004a). Intensification of somatic growth brought about by transgenesis or exogenous hormone treatment has been attributed to alterations in metabolic pathways that support increased myogenesis and efficiency of nutrient utilization (Oakes et al., 2007; Garber et al., 1995); however, nutritional status profoundly influences the expression of growth-related genes, indicating differential roles for somatotrophic ligands as the nutritional condition of the fish varies (Montserrat et al., 2007; Amaral and Johnston, 2011; Bower and Johnston, 2010; Erbay et al., 2003; Chauvigné et al., 2003).

The necessity for rigorous and dynamic regulation of growth imposed by the demands of integrating somatogenesis and muscle growth with other biological processes and environmental parameters has resulted in elaborate interactions between the endocrine effectors involved in this physiology. One such potential interaction may be that between myostatin and the somatotropin-IGF axis, in which synthesis of the active myostatin peptide and the expression of myostatin gene copies have been reported to be moderated by growth hormone (Oldham et al., 2009; Roberts et al., 2004; Biga et al., 2004b; Liu et al., 2003). Transgenic coho salmon (*Oncorhynchus kisutch*) overexpressing *growth hormone* exhibited decreases in both the production of the mature peptide and *myostatin* expression (Roberts et al., 2004). *Myostatin-1* and *-2* have been found to possess sequences in their regulatory domains that are subject to influence by somatotropin (Roberts and Goetz, 2003), suggesting a potential mechanism for transcriptional control. While the transcriptional and translational repression of myostatin by growth hormone may suggest that myostatin functions in myogenesis (Roberts et al., 2004) it may be that the response of myostatin to augmented growth hormone concentrations is secondary to an increase in anabolic activities. The transgenic RBT studied here afforded improved understanding of somatotropin action in the context of myostatin and TGF- β inhibition.

Despite the considerable efforts concentrated on revealing the role of myostatin in teleosts, a definitive role in myogenesis or other regulatory processes has not been resolved. Gaining insight into the function of myostatin in RBT has the potential to reveal the extent of evolutionary conservation or novelty in the myogenic physiology of vertebrate species. The morphological and ecological exigencies imposed on the

teleostean and mammalian clades would likely have influenced the evolution of factors involved in the regulation of an indispensable locomotory tissue such as muscle. To further elucidate the function of myostatin in salmonids, myogenesis was induced in a myostatin-knockdown (PD family) and a myostatin/TGF- β -knockdown (ActRIIB family) line of transgenic RBT through administration of exogenous somatotropin. Myostatin inhibition was accomplished by the overexpression of the pro-domain region of the myostatin protein in the PD transgenic fish, which would presumably bind the bioactive domain and inactivate the peptide. By contrast, myostatin along with related TGF- β molecules were incapable of accomplishing signal transduction in the ActRIIB family as the truncated receptor precluded initiation of the intracellular cascade. Stimulation of growth in the context of myostatin inhibition exclusively, as well as against a background in which myostatin and related TGF- β ligands had been inhibited, enabled discrimination of the function of myostatin and other TGF- β cytokines in teleostean myogenesis. It was anticipated that skeletal muscle growth, the mode of myocyte growth, and expression of myogenic genes in TG fish would exceed that of NTG fish in the event that myostatin, alone or in combination with other TGF- β ligands, function to negatively regulate skeletal muscle development in a basal teleost.

2. METHODS

2.1 *Animals*

Transgenic RBT from two distinct families produced at the East Farm Aquaculture Center in Kingston, RI as previously described (Phelps et al., 2013) were used for this work. Families were selected from a number of those available due to the greater percentage of transgenic individuals within them. The members of the two respective families were F₁ full siblings, approximately 7 to 9 months of age at the outset of the study. The experimental procedures involving these fish were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Rhode Island (AN07-04-031 and AN08-02-012).

2.2 *Fish husbandry*

Fish were fed to satiation 4 to 7 times daily with a commercially available trout feed (Silver Cup, Tooele, UT, USA) through a combination of manual feeding and an automated feeding system regulated by a timer (Sweeney, Welfare, TX, USA). Tanks were supplied with flow through water and supplemental aeration. Water temperature ranged from 7°C to 9°C during the study. Simulated natural photoperiod was maintained by overhead lighting controlled by automated timers, photoperiod was adjusted as necessary throughout the study. Prior to each of the five time points at which length and mass were measured feed was withheld for at least 12 hours to minimize handling stress and resumed within 24 hours of taking the measurements.

2.3 *Constructs and Genetic Screening*

One family carried the activin receptor IIB (ActRIIB) transgene construct, which encodes an isoform of the zebrafish activin IIB receptor in which the encoded protein has been truncated and the intracellular serine-threonine kinase domain is absent, thereby

obstructing signal transduction upon binding of the extracellular domain by myostatin. Use of the zebrafish activin receptor sequence enabled discrimination of transgenic individuals from those with the endogenous RBT activin receptor sequence (Phelps et al., 2013). The other family possessed the pro-domain (PD) transgene construct, in which a premature stop codon had been inserted into the endogenous *myostatin* sequence following the amino-terminal proteolytic cleavage site (Phelps, 2010). ActRIIB and PD families were screened to distinguish TG and NTG animals for the purposes of partitioning them into experimental groups. A small piece of tissue was excised from the caudal fin of each fish while under anesthesia (tricaine methanesulfonate; Western Chemical, Ferndale, WA, USA; 75 mg L⁻¹) to carry out genomic DNA extraction. Tissue was placed in 200 µl of lysis buffer (50 mM L⁻¹ KCl, 10mM L⁻¹ Trizma base pH 8.8, 1.5 mM L⁻¹ MgCl₂, 0.1% Triton X-100) and heated at 100°C for 5 minutes in a thermal cycler (PTC-200, MJ Research, Waltham, MA, USA). Following this, proteinase K (3.2 units, Sigma-Aldrich, St. Louis, MO, USA) was added and the tissue was digested at 55°C for one hour, followed by a 10 minute incubation period at 100°C to denature the enzyme. Centrifugation of the digested tissue at 6,000 x g for 8 minutes (Allegra 25R, Beckman Coulter, Brea, CA, USA) provided a supernatant that was used to run construct-specific 25 µl polymerase chain reactions (PCR). The reactions for the screening of the ActRIIB fish used the following thermal cycler conditions: initial denaturation at 94°C for 1 minute, 36 cycles of denaturation at 94°C for 1 minute, annealing at 63°C for 30 seconds, and extension at 72°C for 1 minute, followed by a period of 10 minutes at 72°C. The conditions for the screening of the PD group were identical with the exception that the annealing temperature was 67°C. The forward and reverse primers for the specific

assays are provided in Table 3. All of the reactions made use of *Taq* DNA polymerase, *Taq* Buffer, and deoxynucleotide solution mix (10 mM each nucleotide) (New England Biolabs, Ipswich, MA, USA) in molecular grade water. The completed reactions were electrophoresed on a 1% TAE agarose gel containing ethidium bromide in 1X TAE buffer. The gels were visualized using a Kodak[®] Gel Logic 100 imaging system (Carestream Molecular, Rochester, NY, USA).

Passive integrated transponder (PIT) tags (Biomark, Boise, ID, USA) were implanted in each of the fish to facilitate identification. Additionally, the NTG fish in both groups had the adipose fin removed to distinguish them from TG individuals. Upon completion of screening the ActRIIB and PD groups were each transferred to one of two 2 m diameter tanks. The TG and NTG fish from each family were housed communally in their respective tanks.

2.4 Experimental treatments

Fish were acclimated to the tanks for a minimum of 48 hours prior to administration of the treatment. Within families, TG and NTG fish were non-selectively chosen for treatment with either recombinant bovine somatotropin, rbST, (Posilac[®], Eli Lilly, Indianapolis, IN, USA) or sesame oil, which represented the placebo treatment. The rbST was injected in a single dosage that produced a final concentration of 50 $\mu\text{g g}^{-1}$ body weight; the volume of Posilac[®] required to achieve this dosage was calculated based upon the mass of each fish individually. Sesame oil volumes were determined in the same manner, using body mass to calculate the volume of oil injected. Treatments were administered under anesthesia (MS-222) by means of a Microman[®] positive displacement pipette (Gilson, Middleton, WI, USA) the tip of which was inserted through a small

incision made in the ventral body surface which penetrated the coelomic cavity to promote delivery and sustained release of the respective treatments. Use of the rbST and placebo treatment generated four groups within each family: rbST-treated transgenic fish (rbST-treated TG), placebo-treated transgenic fish (placebo TG), rbST-treated non-transgenic fish (rbST-treated NTG), and placebo-treated non-transgenic fish (placebo NTG). Each of the eight groups contained 20-25 trout.

2.5 Measurement of growth

Injection of the rbST or sesame oil represented the beginning of the experiment period (day 1), which lasted 84 days. Fork length (mm) and mass (g) of each individual were measured and recorded on day 1 and then at each 21 day interval. The length and weight measurements were used to calculate the condition factor (K) for each individual, according to the formula $K = (Wt \times 100) \div (L^3)$. Specific growth rate for length and mass was calculated for the interval between each successive time point according to the formula $SGR = ((\ln X_1 - \ln X_2) \div 21) \times 100$; where 21 was the number of days between each time point and X was the length or mass. During measurement fish were anesthetized to minimize handling stress. Following collection of the growth measurements the fish were returned to the respective tanks.

2.6 Tissue collection

Following measurement at the final time point (day 84), ten fish from each of the treatment groups (40 fish per family, 80 fish total) were euthanized with an overdose of tricaine methane sulfonate and tissue was collected for RNA extraction and histological sectioning. Using a sterile scalpel, white muscle from the epaxial musculature was excised and transferred to RNAlater (Ambion, Foster City, CA, USA). The stabilized

tissue was stored under refrigeration for approximately 24 hours, followed by transfer to -20°C until RNA was extracted.

A transverse section 10-15 mm in thickness was cut from each fish immediately rostral to the leading edge of the dorsal fin. A piece of tissue no more than 4 cm³ was excised from the epaxial musculature within the section on either side of the dorso-ventral midline. The tissue was placed in Tissue-Tek[®] O.T.C. (Thermo Fisher Scientific, Pittsburgh, PA, USA) embedding compound within tissue cassettes oriented such that cross sections could be cut of the longitudinal fibers of the trunk musculature. The tissue was frozen in 2-methylbutane cooled by liquid nitrogen as previously described (Medeiros et al., 2009) and stored at -70°C.

2.7 Muscle morphology

Histological sections 7 µm thick were cut on a Vibratome UltraPro 5000 Advanced cryostat (Bright Instruments, Huntingdon, ENG). Cross sections were cut from six fish selected from each of the four treatment groups (24 fish per family). The sections were transferred to Poly-L-Lysine coated slides, and stained using Gill's hematoxylin (2%), followed by counterstaining in eosin Y (1%) (Fuentes, 2010). Sections were preserved with Permount (Thermo Fisher Scientific) and a cover slip, and visualized and photographed with a Zeiss LSM 5 Pascal microscope (Thornwood, NY, USA). For each of the six individuals from each treatment group in both families (48 fish total) three distinct cross sections were prepared and stained on individual slides. To preclude observer bias tissue samples were sectioned without knowledge of the origin of the tissue. Images of the histological sections were analyzed using ImagePro Plus version 5.0.1 (Media Cybernetics, Bethesda, MD, USA). One image was generated from

each of the three sections cut for each fish. Within each image three 0.2538 mm² areas of interest were analyzed ($n = 9$ per fish). The areas of interest were distinct, neither touching nor overlapping and free from artifacts and breakages. In performing counts of the individual fibers any fiber contained in whole or part within the defined area of interest was regarded as a single fiber and was included in the total number. The fiber diameter was determined by measuring the shortest distance between the two sides of the fiber at the approximate midpoint of the fiber. Measurement of the fiber diameters was carried out only on fibers that were contained entirely within the area of interest. Similar to sectioning the samples, the fiber number and diameter were measured without knowledge of the transgenic status or hormone treatment of the individual.

2.8 RNA extraction

Some 50 – 100 mg of each preserved muscle sample were removed from RNAlater[®] and transferred to a 2 ml microcentrifuge tube, along with a sterilized 5 mm stainless steel bead and homogenization buffer (RNeasy Mini Kit[®], Qiagen, Valencia, CA, USA), and homogenized with a Qiagen TissueLyser[®] at 25 Hz for three 5 minute intervals. The lysate was centrifuged and the resulting supernatant was applied to silica columns, and the binding, washing, and elution protocols performed in accordance with the manufacturer's instructions; on-column DNase digestion was carried out during the course of extraction with an RNase-Free DNase kit (Qiagen) to remove contaminating genomic DNA. The concentration of the total RNA extracted was measured using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Three 2 µl volumes of the RNA eluate were measured and averaged to provide the RNA

concentration of the sample. The RNA for all samples was diluted to a final concentration of 25 ng μl^{-1} for use as template.

2.9 Amplicon cloning and primer design

Assays measuring the expression of the ActRIIB construct, *myostatin* pro-domain, and *GAPDH* had been established previously in the laboratory (Table 3; Phelps, 2010). The *MyoD* and *myogenin* sequences from RBT were cloned to develop RT-qPCR assays (Tables 2 and 3). Sequence data for *MyoD* (GenBank accession number NM_001124720) and *myogenin* (GenBank accession number Z46912) were utilized to design primer pairs (Integrated DNA Technologies: Coralville, IA). The forward primers were designed such that they contained the palindromic sequence recognized by the restriction enzyme XbaI (5'-TCTAGA-3' Table 2). Similarly, the reverse primers were designed to incorporate the sequence recognized and cut by the restriction enzyme SbfI (5'-CCTGCAGG-3').

Total mRNA was used to produce cDNA utilizing oligo dT and Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (New England Biolabs, Ipswich, MA, USA). This cDNA was used as the template in separate endpoint polymerase chain reactions using the *MyoD* or *myogenin* primer sets, to generate the putative amplicons. The amplicons were resolved on a 0.8% TAE agarose gel containing ethidium bromide.

The amplicon DNA was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions, and quantified as above. 1 μg of the *MyoD* and *myogenin* amplicon DNA, as well as 1 μg of pUC19 vector DNA (New England Biolabs) were digested with XbaI and SbfI (New

England Biolabs), for two hours at 37°C. The amplicon and vector DNA were purified using the QIAquick PCR purification kit.

Ligation of the two amplicons with the vector DNA was performed in separate reactions using T4 DNA ligase. The ligation reactions were incubated overnight at 16°C. Following ligation, chemically competent XL1-blue *E. coli* cells were transformed with either the *MyoD*/pUC19 or *myogenin*/pUC19 plasmids by means of heat shock per the manufacturer's suggestion. Transformed cells were plated on LB agar containing ampicillin at a concentration of 100 µg ml⁻¹, and incubated at 37°C overnight.

Isolated colonies were selected from the plates to inoculate Luria Bertani (LB) broth containing ampicillin (100 µg ml⁻¹), and grown overnight at 37°C and 250 rpm. The plasmid DNA was extracted from the cell cultures using a QIAprep Spin Miniprep kit (Qiagen) in accordance with the manufacturer's instructions. The plasmid DNA was sequenced at the Genomics and Sequencing Center at the University of Rhode Island (URIGSC) using the M13 forward (-20) primer. The presence of the intended clones was confirmed by referencing the sequences against those available in public databases.

2.10 RT-qPCR

The expression of *MyoD* and *myogenin* was determined in the individuals sampled in the ActRIIB and PD families. Transcription of the ActRIIB transgene construct was measured in the samples from growth hormone-treated and placebo TG fish in the ActRIIB; tissue from NTG fish was also assayed to establish absence of the transcript. The assay utilized to analyze *myostatin* expression employed primers that bound the amino-terminal pro-domain region of the transcript, and was therefore unable

to discriminate between native transcripts and those resulting from the pro-domain construct.

Expression assays were performed using single-step RT-qPCR with Brilliant II SYBR Green master mix and reverse transcriptase (Agilent Technologies, Santa Clara, CA, USA) on a LightCycler 480 (Roche Diagnostics, Indianapolis, IN, USA) at the URIGSC. Transcriptional activity was measured in ten individuals from each of the four treatment groups in both the ActRIIB and PD families (40 fish per family) using triplicate 25 μ l reactions that contained 50 ng of total RNA template. Expression of the gene of interest within each sample was normalized to that of *GAPDH*, which was determined using a *GAPDH*-specific assay (Table 3). Potential contamination by genomic DNA was evaluated in triplicate reactions containing RNA template but lacking reverse transcriptase; for each treatment group a pooled RNA template was generated from the ten samples to assess potential contamination in all samples. Triplicate reactions were carried out without the addition of RNA template to ensure reagents were not contaminated. Assay-specific standard curves were generated using cDNA amplicons as the templates for the reactions with copy numbers of the amplicon ranging from 10^1 to 10^8 .

Expression of the ActRIIB transgene construct was assayed on a LightCycler 480 (Roche Diagnostics) using the following conditions: 50°C for 30 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 30 seconds, and 65°C for 90 seconds. The *myostatin* pro-domain, *MyoD*, *myogenin*, and *GAPDH* reaction conditions were identical with the exception of the annealing temperatures, which were 60°C, 62°C, 61°C, and 63°C, respectively, and a 60, rather than 90, second extension period. The potential for

amplification of non-specific sequences was assessed through a dissociation curve analysis at the conclusion of the amplification phase. Following the final cycle of the amplification phase the samples were denatured as the temperature was increased from the respective annealing temperature to 95°C at a rate of 0.11°C second⁻¹, while fluorescence was continuously measured.

2.11 Analysis

Statistical analyses were performed on the length, mass, condition factor, specific growth rate (length and mass), histological, and gene expression data using SAS, version 9.3 (SAS Institute, Cary, NC, USA). Prior to carrying out the analyses the data were evaluated for normality using the Shapiro-Wilk test or Kolmogorov-Smirnov test. Normally distributed data sets were analyzed using a factorial ANOVA that assessed the impact of both transgenesis and rbST administration, along with any putative interaction between the two. Subsequently, all of the data were analyzed with four separate one-way ANOVAs or equivalent nonparametric tests on the basis of genotype and treatment to validate the results of the factorial ANOVAs and better discriminate differences within and between both variables. Length, mass, and condition factor data that were not normally distributed were transformed using the natural log. Data that remained non-normal following natural log transformation were partitioned by both genotype and hormone treatment creating four data subsets in which a single variable was evaluated. These subsets were evaluated with the Mann-Whitney statistic to which, when dictated by the characteristics of the dataset, the Monte Carlo simulation was added. The necessity for such an approach was directed by the severity of the departure from normality within particular data sets. It permitted only direct comparisons between two

groups on the basis of division by either genotype or hormone treatment, thereby precluding statistical evaluation of any putative interaction. Any potential interaction between genotype and hormone treatment could be inferred by comparison of the four Mann-Whitney tests employed for these particular datasets, though any such interaction could not be statistically validated. The values provided are mean $\pm$ standard error, and all differences denoted as significant are at the level of $p < 0.05$ or less.

3. RESULTS

Fish in both the ActRIIB and PD families tolerated the respective treatments without incident, and were absent any conspicuous adverse health effects at the conclusion of the trial period. All fish fed actively throughout the study and displayed no evidence of infection or disease as evaluated by external examination. Several individuals were lost over the course of the study due to complications unrelated to the experimental procedures. The musculature and internal organs of individuals euthanized to obtain tissue for assays appeared to reflect good health.

3.1 Analyses of morphological parameters and specific growth rate

TG fish in the ActRIIB family were significantly shorter than their NTG siblings receiving the same treatment, i.e. rbST or placebo, at the beginning of the study; however, there was no significant difference between the treatment groups within either the TG or NTG genotypes (Fig. 1). While fish within the same genotype did not differ significantly in mass between treatments at this time, the placebo TG fish weighed significantly less (98.11 ± 5.38 g) than did the placebo NTG fish (115.94 ± 5.89 g); no difference was found between genotypes in the rbST-treated fish (Fig. 2).

At all subsequent time points the TG rbST-treated and placebo ActRIIB fish remained significantly shorter than NTG fish within the same respective treatment group. Similarly, the body mass of the TG ActRIIB fish was significantly less than that of the NTG fish when genotypes were compared within the same treatment group. Beginning at time 2 administration of rbST produced significant differences in the fork length and mass of treated fish compared to those receiving the placebo. ActRIIB TG fish receiving rbST were significantly longer (20.08 ± 0.41 cm) than placebo TG fish (18.92 ± 0.34 cm),

while no such difference was found in the lengths of NTG fish (Fig. 1). The rbST-treated fish of both genotypes were significantly longer than placebo fish of the same respective genotype at all time points beginning with time 3 (Fig. 1). Exogenous hormone yielded significantly greater body mass within both genotypes from time 2 throughout the duration of the study (Fig. 2). Genotype rendered a significant effect on condition factor for the entirety of the study, as the condition factor of the ActRIIB TG fish receiving hormone and placebo treatments was significantly higher than the NTG fish within the same treatment group throughout (Fig. 3). Hormone treatment produced a significantly higher condition factor within the ActRIIB TG fish at time 4 only, at all other time points there was no difference between treatment groups in the TG ActRIIB fish. By contrast, rbST-treated NTG fish possessed a significantly higher condition factor than their placebo-treated siblings at all time points beginning with the second (Fig. 3). The interaction between genotype and hormone treatment did not prove significant at any point.

The SGR evaluated for both the fork length and mass revealed that hormone treatment yielded a predominant influence on growth (Figs. 7 and 8). With respect to length, the SGR was significantly greater in the rbST-treated fish, irrespective of genotype; however, within the ActRIIB TG fish the SGR was not different between treatments from time point four to five (Fig. 7). The SGR measured for mass was similarly significant within the rbST-treated fish at all intervals but the last, i.e. between time points four and five, when no difference was found between treatments in either genotype. Genetic background, i.e. TG or NTG, failed to generate a differential influence on the SGR of either length or mass, with a single exception. The SGR of mass in the

rbST-treated TG ActRIIB fish was significantly less than that of the rbST-treated NTG fish between the first and second time points; at all other intervals no difference in growth rate was found between the TG and NTG fish receiving the same treatment in the ActRIIB family. The SGR of both length and mass did, however, decrease within the rbST fish over the course of the experiment, regardless of genotype

The fork length, mass, and condition factor of PD TG fish receiving hormone and placebo treatments did not differ from those of similarly treated NTG fish at the outset of the study (Figs. 4-6). At such time no differences were found between treatment groups within either the TG or NTG fish of the PD family. The length, mass, and condition factor of the rbST-treated TG and NTG fish emerged as significantly greater than those of the placebo fish within the same genotype at time point 3; some parameters had achieved significance as early as time point 2. At no point were any of these metrics significantly different between the two genotypes for fish in either the rbST or placebo groups; exogenous somatotropin therefore consistently proved to generate a significant effect which myostatin inhibition did not. The interaction between genotype and hormone treatment was not significant for fork length, mass, or condition factor at any point in the experiment.

The growth rate, measured as a function of both length and mass, was influenced by rbST throughout the experiment, though not by genetic composition. The SGR for fish length was significantly greater in the rbST-treated fish of both TG and NTG fish throughout. Similarly, increases in mass were significantly greater in the rbST-treated fish of both genotypes; however, a difference in the SGR for mass was observed at interval 4 in the NTG fish but not in the TG fish. For fish within the same treatment

group there were no significant differences in the SGR of either length or mass between TG and NTG fish at any interval between time points. As in the ActRIIB family, the growth rate of the rbST-treated fish decreased as the experiment proceeded.

3.2 Analyses of histological sections

Myofiber development in epaxial musculature of the ActRIIB fish was impacted by both the transgene and rbST treatment. The number of myofibers was not significantly different between the rbST-treated (89.4 ± 1.7) and placebo (88.1 ± 2.1) TG fish at the time of sampling (Fig. 11). In the NTG fish, however, those treated with rbST had a significantly greater number of fibers (93 ± 2) than those receiving the placebo (80.3 ± 1.3); the significant interaction term provided by the factorial ANOVA indicating that the proliferation of myofibers resulting from rbST treatment was dependent upon the genetic background, i.e. TG or NTG, into which it was introduced. Moreover, the number of myofibers in the rbST-treated TG fish (89.4 ± 1.7) was not different from that of the rbST-treated NTG fish (93 ± 2).

Myofiber diameters in both the TG and NTG fish receiving rbST were significantly smaller than those of the placebo fish of the same genotype within the ActRIIB family. Additionally, the fiber diameters of the rbST-treated TG fish ($37.29 \pm 0.46 \mu\text{M}$) significantly exceeded those of the rbST-treated fish NTG ($35.78 \pm 0.44 \mu\text{M}$) (Fig. 12). However, myofibers in the fish receiving the placebo were significantly smaller in the TG fish ($38.44 \pm 0.41 \mu\text{M}$) than those of the NTG fish ($43.82 \pm 0.50 \mu\text{M}$). The inability to carry out a factorial ANOVA analysis on the data due to the non-normal distribution precluded statistical assessment of any putative interaction between genotype and treatment. The differential response of the genotypes to the hormone treatment and

placebo did, however, suggest an interaction between the variables may have been present; though this could not be statistically validated. Notably, the placebo NTG fish possessed the largest diameter fibers among the four treatment groups.

Myofiber number in the PD fish was influenced by both genotype and hormone treatment, as it was in the ActRIIB fish (Fig. 13). A significant interaction existed between the variables; the administration of rbST differentially influenced muscle cell proliferation in the two genotypes. Within the TG fish no significant difference in the number of myofibers was found between the fish treated with rbST (64.1 ± 1.8) and those receiving the placebo treatment (63.6 ± 2.1) (Fig. 13). NTG fish, however, that were treated with rbST had a significantly greater number of myofibers (85.4 ± 1.8) than NTG fish receiving the placebo (61.6 ± 1.2). The number of fibers in the rbST-treated TG fish was significantly less than that in the NTG fish receiving the hormone treatments; in contrast no difference was found between genotypes in the fish receiving the placebo.

In both the TG and NTG fish those individuals receiving rbST had significantly smaller diameter fibers than those receiving the placebo treatment. Additionally, no significant difference existed between the fiber diameters of the rbST-treated TG fish ($41.60 \pm 0.67 \mu\text{M}$) and the rbST-treated NTG fish ($39.08 \pm 0.57 \mu\text{M}$) (Fig. 14). The placebo TG fish did, however, possess significantly smaller diameter fibers ($47.70 \pm 0.62 \mu\text{M}$) than the placebo NTG fish ($50.37 \pm 0.69 \mu\text{M}$). The non-normal distribution of the data set precluded factorial ANOVA analyses and the evaluation of any interaction between genotype and hormone treatment.

3.3 Expression analyses

Expression of the transgene construct in the ActRIIB rbST-treated (0.55 ± 0.09) and placebo (0.79 ± 0.09) TG fish was not significantly different (Fig. 15). The PCR assay was designed to detect only the construct, i.e., zebrafish (*D. rerio*) activin IIB receptor sequence that codes for a truncated receptor protein; no construct was detected in NTG fish.

Expression of the *myostatin* pro-domain in the ActRIIB family did not differ between genotypes for fish treated with rbST, though it was significantly higher in the TG fish receiving the placebo (1.34 ± 0.25) when compared to the NTG fish in the same treatment group (0.52 ± 0.13) (Fig. 16). The assay was specific to the endogenous *myostatin* pro-domain and therefore reflected expression of immature form of the peptide. *Myostatin* pro-domain expression was not different between the rbST-treated and placebo fish of either genotype.

Transcription of the muscle regulatory factor *MyoD* was significantly greater in the rbST-treated fish than that in placebo-treated fish of both genotypes. While the expression was not significantly different between the rbST-treated fish of different genotypes, *MyoD* expression in the placebo TG fish (1.26 ± 0.08) was significantly greater than that of the placebo NTG fish (0.87 ± 0.14) (Fig. 17). The non-normal distribution of the *myogenin* expression in the ActRIIB family precluded evaluation of any putative interaction between genotype and hormone treatment; however, no significant differences were found in *myogenin* expression between hormone-treated and placebo fish within either genotype, or between TG and NTG fish receiving rbST or placebo (Fig. 18).

In PD fish transcription of the *myostatin* pro-domain was significantly greater in both the TG rbST-treated (3.19 ± 0.79) and placebo (1.73 ± 0.18) fish than in the NTG fish within the same respective treatment group (Fig. 19). Within the two genotypes expression levels did not differ significantly between treatment types. The assay measured levels of the endogenous myostatin transcript; therefore transcript levels in the NTG fish were considered indicative of basal expression and the significantly greater expression in the TG fish were regarded as a function of the activation of the transgene construct.

The expression of *MyoD* in the PD fish was differentially influenced by both genotype and hormone treatment (Fig. 20). The rbST-treated TG fish (0.98 ± 0.12) exhibited significantly higher expression than the placebo TG fish, while a difference between the treatment groups was absent in the NTG fish. TG fish additionally exhibited significantly higher *MyoD* transcription than their NTG siblings of the same treatment type.

Myogenin expression in the PD fish was not significantly different between the rbST-treated TG (1.12 ± 0.13) and placebo TG (1.39 ± 0.16) fish. Expression in the rbST-treated NTG fish (1.08 ± 0.08) was significantly lower than that of the placebo NTG fish (1.76 ± 0.14). No significant differences were found between fish of differing genotypes receiving the same treatment type (Fig. 21).

4. DISCUSSION

Myostatin has been established as a potent antagonist of mammalian muscle growth and development (McPherron and Lee, 1997), functioning to inhibit mitogenic and terminal maturation programs in myogenic precursors (Thomas et al., 2000; Langley et al., 2002; Ríos et al., 2002) along with protein synthesis pathways in skeletal muscle tissue (Taylor et al., 2001). The conservation of myostatin physiology in other vertebrate taxa remains under investigation, and insight into myostatin function in rainbow trout facilitates understanding of the evolution of myogenic processes within vertebrates. Inhibition of myostatin exclusively, as well as alongside related TGF- β molecules, afforded a unique opportunity to more clearly articulate the function of these endocrine effectors in teleostean myogenesis. The somatogenesis induced by the administration of rbST provided a novel context in which to better understand the function and regulation of myostatin and TGF- β molecules in the muscle growth of a basal teleost. The transgenic inhibition of these TGF- β proteins indicated that they do not operate to restrict myogenesis in trout as they do in mammals. While members of the TGF- β family other than myostatin that signal by means of the activin IIB receptor do appear to influence axial morphology and myocyte proliferation, none of the peptides examined appear to function prominently in the myogenesis of RBT.

The TG individuals in the ActRIIB family were defined by a unique phenotype relative to their NTG siblings, a feature not found in the PD family. The high condition factor that was the hallmark of the ActRIIB TG fish throughout the duration of the study resembled that observed by Phelps et al. (2013) in P₁ and F₁ generations expressing the same truncated activin receptor construct. Coincident with the greater condition factor in

the ActRIIB TG fish were a reduced body mass and shorter fork length, that translated to a more compact morphology. The TG and NTG fish within the ActRIIB family treated with rbST had a significantly greater fork length and body mass than placebo-treated siblings of the same respective genotype. Within both treatment groups, however, the TG fish remained significantly shorter and exhibited a smaller body mass than the NTG individuals. The morphological differences between the TG and NTG ActRIIB fish contrasted with the relative uniformity observed in the PD family. The significant increases in fork length, body mass, and condition factor in the PD family were the result of administration of rbST; the genetic backgrounds, i.e. TG or NTG, into which the hormone was introduced failed to generate differences in growth parameters.

The incongruent morphology of TG individuals of the ActRIIB and PD families corresponded to both the TGF- β ligands being inhibited in the two TG lines, and the respective mechanism of inhibition. The distinct phenotype observed in the TG ActRIIB fish in which TGF- β s signaling through the activin IIB receptor were obstructed was not shared by the TG fish of the PD family, in which myostatin alone was inhibited. Moreover, in the TG fish of both families a conspicuously muscular appearance reminiscent of the “double muscled” phenotype characteristic of mammalian species with myostatin deficiencies, was absent. Several domesticated mammalian species, including breeds of cattle (McPherron and Lee, 1997; Kambadur et al., 1997; Muroya et al., 2009) possess nonfunctional myostatin alleles attributable to various mutations. Similar muscularity in whippets (Mosher et al., 2007) and Texel sheep (Clop et al., 2006) have additionally revealed decreased myostatin signaling as the causative agent. Transduction of myostatin signaling to intracellular effectors is dependent upon binding of the

transmembrane activin receptor complex (Lee and McPherron, 2001; Derynck and Zhang, 2003). Experimental manipulation of the activin type II receptor yielded mice with significantly increased skeletal muscle, resembling that found in myostatin-null models (Lee et al., 2005; Lee and McPherron, 2001). Importantly, muscle mass was further enhanced in the complete absence of myostatin through the inhibition of related TGF- β ligands including activin A (Lee et al., 2010), suggesting the participation of additional effectors alongside myostatin in the regulation of mammalian muscle physiology (Lee, 2007).

In stark contrast to the exceptionally muscular phenotype produced by simultaneous inhibition of myostatin and related TGF- β ligands, the TG ActRIIB fish failed to exhibit a significant increase in myogenesis. Indeed, the TG fish in the ActRIIB family experienced an alteration in morphology and failed to achieve the body length and mass attained by NTG siblings. One or more of the endocrine effectors utilizing the receptor complex of which the activin type IIB hemireceptor is a component appears to be integral to the myogenic, and even somatogenic, homeostasis that enables typical growth in these fish.

The specific inhibition of myostatin in the PD family suggests that the involvement of myostatin in the alteration to morphology and axial development observed in TG ActRIIB fish is minimal. The PD TG fish diverged only minimally from the phenotype of their NTG siblings, unlike the significant disparity observed between genotypes in the ActRIIB family. Notable, however, was the finding that in both families the TG fish consistently remained smaller in length and body mass than NTG siblings. While the difference was more pronounced in the ActRIIB TG fish, it is nonetheless of

interest that both myostatin-specific and more generalized TGF- β inhibition facilitated a change in morphology. Comparison of the ActRIIB and PD TG individuals suggests participation of undetermined TGF- β effectors in both the axial and skeletal muscle development of basal teleosts. However, the absence of intensified muscle growth in both families of fish indicates that myostatin and other TGF- β endocrine effectors investigated here may not function specifically in myogenic physiology, nor are they likely vigorous antagonists of myogenic growth and regulation in salmonids as they are in mammals.

Deletion of *Gdf11* in mice has been reported to produce alterations to the body axis and hindlimbs due to homeotic anomalies (McPherron et al., 1999), and though exploration of any such change was not within the scope of this investigation, gross anatomy and cursory observation upon dissection of individuals did not indicate similar modifications. Were such divergent morphology found in trout it may be explained by the failed signal transduction resulting from GDF11 binding by the truncated activin IIB receptor. We have previously reported (Phelps et al., 2013) on a striking phenotype present in members of the P₁ generation of ActRIIB trout that was manifestly absent in the F₁ generation. The lateral epaxial and hypaxial musculature of these individuals exhibited localized muscling present in only some of the somites along the body axis; this phenotype additionally served to support the expression and functionality of the transgene construct. The ActRIIB TG fish utilized in the current work were from the F₁ generation, and did not possess the localized enhanced musculature that was predominant in the P₁ generation. The offspring of the individuals originally injected with the ActRIIB transgene construct have therefore been demonstrated to experience differential

body morphology with a concomitant loss of the regionalized musculature present in their progenitors. The effects of transgenesis on the F₁ generation evidence a consistent role for TGF- β peptides in the growth and development of the skeletal muscle, as well as other tissues. While the unique phenotype observed in the P₁ generation may be attributed to epigenetic expression subsequent to injection with high concentrations of the construct, or differential expression owing to unknown factors, the establishment of the truncated receptor in the subsequent generation produces a singular, albeit alternative, phenotype. The P₁ generation was also defined by unique phenotypes in each of the TG individuals, which contrasted with the relatively uniform phenotype observed in all of the F₁ ActRIIB TG fish in this study.

Specific growth rate was not influenced to any discernible degree by the inhibition of myostatin, either alone or in concert with other TGF- β s, further supporting a limited role for myostatin in muscle growth, at least for the duration of the life cycle examined here. The fish in both families had not reached sexual maturity, and were studied at a stage of life in which they would have been expected to experience accelerated growth. The ability of the ActRIIB TG fish to sustain equivalent growth rates with their NTG siblings, despite being consigned to suboptimal body size early in ontogeny, suggests that TGF- β inhibition constrains growth, but that somatogenesis is sufficiently plastic to enable growth rates proportional to their reduced size when permissible conditions allow, i.e. the introduction of exogenous hormone. Further, the morphological differences observed between these fish are established early in ontogeny by the deficiency in TGF- β signaling, and persist despite augmentation through extrinsic growth hormone. The somatotrophic axis remains among the foremost mediators of

vertebrate growth (Björnsson, 1997) and has remained remarkably well conserved throughout vertebrate evolution with respect to many of its principal endocrine effectors, tissues, and receptors (LeRoith et al., 2001). Somatic growth in the rbST-treated individuals in both families studied here supports the ubiquity of this vertebrate physiology and the definitive role it has in fish growth.

Considerable effort has been invested in illuminating and exploiting the somatotrophic axis in a number of commercially valuable fish species in an attempt to encourage greater growth potential (De-Santis and Jerry, 2007). Exogenous recombinant hormone has been demonstrated to promote growth in salmonids (Agellon et al., 1988; Garber et al., 1995; Schulte et al., 1989; McLean et al., 1997) as well as other species (Peterson et al., 2004; Wille et al., 2002). Growth hormone transgenesis has also succeeded in stimulating significant growth in salmonids (Devlin et al., 1995; Raven et al., 2012). The increase in growth rate is associated with concomitant modifications to the expression profiles of a suite of genes concerned with metabolic and growth physiologies (Raven et al., 2008; Overturf et al., 2010; Raven et al., 2012), relying on a redistribution and alternative utilization of metabolites to provision the anabolic processes of growth (Leggatt et al., 2009). Both the specific growth rate and the increased size of the fish that received rbST in the ActRIIB and PD families support previous findings. However, the failure of TG individuals to achieve a skeletal muscle phenotype and growth surpassing that of NTG fish demonstrates the absence of a highly muscle-specific or substantial role for either myostatin or select TGF- β proteins inhibited here in the myogenesis of these fish. Were these ligands prominently involved in muscle development, the placebo TG individuals would presumably have exhibited growth

beyond that found in their NTG siblings. Additionally, TG fish receiving hormone treatments might be expected to exceed their NTG sibling in some form of an additive effect, however, neither event occurred. Instead, the specific growth rates of TG and NTG fish in both families were equivalent within the respective treatment groups throughout the duration of the study.

Characteristic of both families was an accelerated growth rate induced by the anabolic effects of rbST administration, regardless of myostatin or TGF- β inhibition. The convergent effects of growth hormone transgenesis and selective breeding focused on enhancing growth performance have revealed that essentially identical pathways and regulatory programs are responsive to these two disparate mechanisms of somatogenic augmentation (Devlin et al., 2009; Overturf et al., 2010; Raven et al., 2012). In such studies the domesticated breeds in which maximal growth potential had been encouraged typically grew at rates, and in a manner similar, to TG fish (Devlin et al., 2001); moreover, additional growth was not realized in growth hormone TG fish receiving exogenous hormone treatment (Raven et al., 2012). The authors suggested that the capacity for somatogenesis had been nearly exhausted by either artificial selection or transgenesis in nearly equivalent terms. In the current study, the ability of rbST to similarly stimulate growth in the TG and NTG fish, coupled with the fact that TG fish receiving a placebo treatment neither surpassed NTG fish nor approached the growth of hormone treated fish suggests that myostatin and TGF- β inhibition do not translate to the bolstered growth potential observed in growth hormone TG fish. Regarded collectively, the fish used in this study should not be considered to have achieved the maximal growth permissible by genetic selection as they were bred from stocks not previously selected for

accelerated growth or commercial production. Additional support for the critical function of TGF- β s in somatic development and patterning is provided by the more uniformly compact body shape in the ActRIIB TG fish that is lacking in the PD TG fish and the difference in the condition factor between TG and NTG fish within the ActRIIB family. Growth hormone TG salmon exhibit somatogenesis in a homogeneous fashion throughout the body, without growth concentrated in a particular tissue or region of the body (Devlin et al., 2004); the compressed morphometrics of the ActRIIB TG fish here were similarly uniform. As in the growth hormone TG fish, the ActRIIB TG fish exhibited alterations throughout the body that were likely established early in ontogeny as a function of constitutive endogenous expression of the truncated receptor, and maintained as the fish aged. Within the rbST-treated group the increased condition factor of the TG fish relative to the NTG fish that persisted throughout the study demonstrated that the compact body form was maintained even as the TG fish grew at a rate equivalent to that of the NTG fish. The preservation of this morphology suggests endocrine and paracrine or autocrine functions of the TGF- β s being inhibited by the nonfunctional receptor as the unique body form is proportional and absent any localized anatomical anomalies.

The myocyte composition of the epaxial musculature was not markedly altered by the inhibition of myostatin or TGF- β effectors in either the ActRIIB or PD families. The fibers examined were derived from the glycolytic somatic musculature within the body mass and were representative of the epaxial phenotype. However, increased hyperplastic growth in the TG fish of the ActRIIB family contrasted sharply with the distinct paucity of hyperplasia to an equivalent degree in the TG fish of the PD family. As the inhibition

of myostatin exclusively in the PD family failed to generate a phenotype marked by the more extensive hyperplasia seen in the TG ActRIIB fish, it would appear that TGF- β molecules other than myostatin signaling by means of the activin IIB receptor are necessary to impede cell proliferation. Hyperplastic growth has previously been demonstrated in our lab in RBT expressing the ActRIIB transgene construct (Phelps et al., 2013) as well as those overexpressing the myostatin/TGF- β antagonist follistatin (Medeiros et al. 2009). While increased hyperplasia has been reported in zebrafish over expressing the PD (Xu et al., 2003) this species has a determinate growth mode that differs from the indeterminate growth found in salmonids.

The degree of hyperplasia observed in the ActRIIB fish was, notably, not intensified by the administration of rbST. Proliferation in the NTG rbST-treated fish of the ActRIIB family did not differ from that of the TG fish in the rbST-treated group. It may be that the proliferative capacity of the TG fish had been fully exploited by inhibition of a TGF- β effector regulating myoblast proliferation in these fish. Exogenous hormone administration, in the event of the tissue having fully attained its mitotic capability and thereby depleting its proliferative ability, would fail to generate hyperplasia beyond that which had already occurred. If the myoblasts had exhausted their mitotic potential through the unrestrained proliferation permitted by TGF- β deregulation they would presumably move towards terminal differentiation. This does not, however, account for the inability of rbST to elicit an increase in hyperplasia in the TG fish of the PD family. The absence of a difference in myofiber number in placebo-treated TG and NTG PD fish suggests that myostatin is not the primary mediator of cellular proliferation. However, overexpression of the pro-domain of the endogenous

myostatin protein may perhaps impinge upon the accepted mitogenic actions of growth hormone in some fashion as the rbST-treated TG fish did not exhibit the myofiber proliferation found in the rbST-treated NTG fish. The hyperplasia found in the rbST-treated NTG fish of the PD family was not present in the TG fish receiving hormone treatment. There exists the possibility of an unknown interaction between the myostatin pro-domain and an endocrine factor of the somatotrophic axis or other ligand responsible for stimulating proliferation. In this way myogenesis would proceed normally but would not profit from the cellular proliferation afforded by somatotropin. The absence of significant hypertrophy in the TG PD fish suggests that myostatin is not antagonistic to the anabolic protein synthesis pathways involved in somatogenesis. Additionally, the significantly larger cells in only the rbST-treated TG fish and not the placebo TG fish of the ActRIIB family similarly suggests that other TGF- β ligands do not antagonize protein accretion in trout. While it may be anticipated that an inverse relationship would exist between myofiber number and diameter, as an increase in fiber number typically requires individual fibers to be smaller in diameter, no such distinct trend was consistently found in either family.

Myostatin has been clearly demonstrated to constrain proliferation (Thomas et al., 2000) and differentiation (Langley et al., 2002; Ríos et al., 2002) in developing skeletal muscle in mammals. It has additionally been found to suppress protein anabolism in these cells (Taylor et al., 2001; Trendelenburg et al., 2009), entrenching its function as a myogenic inhibitor. While the myostatin-deficient phenotype has been established *in vivo*, many of the investigations concerning the mechanism of action have been derived from *in vitro* findings. The physiology of myostatin regulation within the organism is

doubtless inordinately more complex; elevated myostatin levels have conversely been found to facilitate viability of cultured myocytes (Ríos et al., 2001). Previously believed to restrict myogenesis by halting the recruitment and perpetuation of myosatellite cells (McCroskery et al., 2003), myostatin is currently understood in mammals to function at the level of the differentiated myofibers themselves by means of inhibiting protein synthesis (Wang and McPherron et al., 2012; Lee et al., 2012; Amthor et al., 2009). The TG fish studied here demonstrate the influence of myostatin and related TGF- β s on muscle. However, it remains unclear whether it is the myotubes or immature myoblasts that are the recipients of such actions. The function of myostatin in trout appears to differ from that in mammals. *In vivo*, undetermined TGF- β molecules appear to inhibit myoblast proliferation whereas myostatin alone does not, contrary to conclusions from investigations utilizing trout myoblast cultures (Seiliez et al., 2012). The disruption of protein synthesis by myostatin found in mammalian cells is not present in trout and, further, a profound shift toward a growth mode dominated by hyperplasia or hypertrophy was not found. The increased hyperplasia observed in the ActRIIB TG fish occurred with only a moderate change in fiber dimensions. Hyperplasia and hypertrophy are present throughout ontogeny in salmonids, serving as the predominant growth mode to varying degrees throughout ontogeny (Johnston et al., 2011). Stratified and mosaic hyperplasia collectively lead to somites populated by a heterogeneous collection of fibers (Rowlerson et al., 1995; Johnston et al., 2003; Rowlerson and Veggetti, 2001). The disruption of myostatin signaling did not appear to lead to a deviation from this mode of growth, and the hyperplasia found in the TG ActRIIB fish failed to translate to a significant increase in muscle mass presumably due to the same enhancement of cross-

sectional surface area observed previously (Phelps et al., 2013). The change in surface area that is secondary to the altered morphometrics would enable an increase in myofiber number without a requisite increase in total muscle mass. The substantial role of myostatin in regulating myocyte proliferation and dimensions in mammals that was not observed in teleosts may be attributed to the determinate growth experienced by mammals. The evolution of a finite size and restricted growth potential in these vertebrates may necessitate a stringent regulation of myogenesis not found in species such as salmonids that possess indeterminate growth modes.

The intricate physiology of myogenesis is regulated by myriad genes and endocrine effectors, the muscle regulatory factors, MRFs, foremost among them; the principal mediators of proliferation and differentiation being MyoD and myogenin, respectively. In the current report *MyoD* expression was revealed to be responsive to regulation by growth hormone as well as myostatin. In mammalian myogenesis, myostatin activity has been demonstrated to inhibit *MyoD* expression in myocyte culture (Langley et al., 2002). However, the transcriptional response in the trout studied in this work indicated a decoupling of *MyoD* expression, presumably one of several paralogues, from the myogenic functionality of the peptide. Up regulation of *MyoD* in the TG individuals in both of the families was found, exceeding that in NTG fish. The presence of a similar transcriptional response to both strategies of myostatin inhibition demonstrates a myostatin-specific suppression of *MyoD* mRNA that does not require related TGF- β peptides. Growth hormone administration similarly produced an increase in *MyoD* activation. Notably, the significant increase in *MyoD* transcription in TG fish was not associated with greater body size or specific growth in the TG fish; rather, TG

fish with greater *MyoD* mRNA concentrations experienced growth rates parallel to NTG fish that had received identical treatments, and did not achieve a larger ultimate size. The up regulation of this MRF failed to increase muscularity or somatogenesis, which is corroborated by the absence of a correlation between myocyte quantity and *MyoD* expression in both families. While increased *MyoD* expression was associated with instances of accelerated growth and superior mass, it does not appear to be the factor motivating such augmentation, but instead a consequence. The regulation of *MyoD* by myostatin in rainbow trout thus appears evolutionarily conserved, at least to a degree, with that in mammals, in that *MyoD* regulation is subject in some fashion to regulation by myostatin. The initiation of myogenic programs and transcriptional activation directed by this MRF, however, does not appear to have been conserved as increased *MyoD* expression did not correspond to enhanced muscle growth.

Myogenin expression failed to differ significantly between most groups, and may reflect a similar decoupling of this MRF from the myogenic activities of fish muscle. Within the ActRIIB family expression was not different between any of the fish, neither genotype nor treatment regime eliciting differential activity. Minimal variation in mRNA concentrations was similarly found within the PD family; the placebo NTG fish exhibited greater transcription than rbST-treated NTG fish. The current data preclude a definitive conclusion regarding the decreased *myogenin* transcript concentrations in some groups of fish and not others. The absence of significant changes in *myogenin* transcription in two families, each expressing a distinct inhibitory construct, suggests that myostatin and TGF- β are not dominant regulators of this MRF. The generally accepted functions of *MyoD* and *myogenin* in the processes of proliferation and determination in myogenic

cells (Pownall et al., 2002) were not supported by the transcriptional activities of these genes in either the ActRIIB or PD family.

Myostatin expression in the fish in both families raised the possibility of autoinhibitory regulation, as well as the potential that myostatin may be requisite for fully functional myogenesis. While a significant increase in *myostatin* expression was anticipated in the PD TG fish due to the presence of the transgene construct significantly increased expression was also detected in ActRIIB placebo TG fish relative to the NTG placebo fish, neither of which possessed the pro-domain transgene. Disrupted myostatin signaling in the ActRIIB TG fish might interfere with an autocrine or paracrine feedback mechanism in the myocytes necessary for interpreting concentrations of the bioactive myostatin peptide; thus resulting in intensive activation of *myostatin* as the signaling cascades initiated by the protein are halted and the cells are unable to detect protein concentrations. In such an event the acknowledged endocrine actions of myostatin may be expanded to include paracrine and/or autocrine mechanisms, as has been previously observed (Ríos et al., 2004). Moreover, while the TG ActRIIB fish were consistently confined to a body size significantly smaller than that of their NTG siblings, the TG PD fish exhibited only a slightly smaller length and mass throughout. While this difference was not significant in the PD TG fish it could suggest that myostatin is in part responsible for rendering the NTG body form, though not to the extent that are related TGF- β molecules. Contrasted with the more radical morphological deviation of the ActRIIB transgenics in which additional TGF- β ligands were interfered with, it proposes a diminished role for myostatin in maintaining non-transgenic myogenesis, and a more arresting necessity of related TGF- β s in muscle development.

The expression of *myostatin* was noticeably not down regulated in the faster growing rbST-treated fish. In TG and NTG fish in both families *myostatin* expression in the hormone-treated individuals was not significantly different from that of the placebo fish within the same respective genotype. The absence of a down regulation of *myostatin* in the context of elevated somatogenesis is contrary to findings in other teleosts (Roberts et al., 2004; Biga et al., 2004b; Gahr et al., 2008) and humans (Oldham et al., 2009; Liu et al., 2003). The interaction between myostatin and growth hormone in these studies was characterized by diminished myostatin peptide concentrations in the presence of increased growth hormone concentrations. In the current work, no such interaction between growth hormone and *myostatin* transcript concentrations was observed; however, it must also be acknowledged that while the expression of *myostatin*, and that of other genes under study, was evaluated this does not absolutely reflect the translation of these mRNAs or the functional protein concentrations present. The discordant findings pertaining to somatotropin effects on myostatin are representative of the efforts still very much in progress to ascertain the precise physiology of myostatin in teleosts.

Ambiguous and diametrically opposing findings for myostatin function have been documented (Gabillard et al., in press), manifesting the intrinsic complexity and subtlety of regulatory mechanisms. Much of the difficulty in fully resolving the workings of myostatin may be attributed to its nearly ubiquitous expression in RBT and many other teleost species, which contrasts with the muscle-specific expression found in mammals (Delgado et al., 2008; Rodgers and Garikipati et al., 2008). Further impeding understanding is the presence of multiple isoforms of genes, myostatin and the MRFs included, due to tetraploidization of the salmonid genome (Jaillon et al., 2004). Multiple

gene copies have enabled differential spatial and temporal expression of these genes across diverse tissues and throughout ontogeny (Macqueen and Johnston, 2008; Rescan et al., 1995; Rescan et al., 2001; Rescan et al., 1999; Delalande and Rescan, 1999; Roberts and Goetz, 2003; Gabillard et al., in press). Indeed, the absence of a clearly defined association between the expression of *MyoD* and *myogenin*, and increased somatogenesis presented here may be a function of analysis of MRF paralogues not involved in myogenesis, or of the inhibition of a *myostatin* or other TGF- β paralogue which is not critical to muscle regulation and development. Certainly the presence of multiple myostatin genes in salmonids (Østbye et al., 2001) represents both a challenge to the study of all of these genes simultaneously when employing a transgenic inhibitory mechanism, as well as an opportunity for these multiple myostatin genes to serve disparate physiological functions. The myostatin protein inhibited in the PD family, myostatin 1, is the primary form of the peptide present in RBT, and Thies et al. (2001) have reported that binding by the pro-peptides of the corresponding protein within the TGF- β is highly fidelitous. The myostatin pro-domain overexpressed in the PD TG fish would therefore presumably effectively bind to and inhibit myostatin 1, though likely not other forms of myostatin. Protein binding assays that could confirm the ability of the myostatin pro-domain to re-bind the bioactive molecule and inactivate it would help to establish with certainty that the absence of increased musculature in the PD TG fish was not due to a failure in the mechanism employed here to inhibit myostatin. Additional efforts, including examination of all paralogues of these genes, will be required to more fully understand the participation of myostatin in teleostean myogenesis. *In vivo*

investigations are more likely to yield physiologically relevant results as they will be able to capture the diversity of regulation present in the intact organism.

The concurrent inhibition of myostatin, both exclusively and in conjunction with related members of the TGF- β family of endocrine factors, in TG rainbow trout afforded the unique opportunity to improve the understanding of myogenic physiology in a basal teleost. Elucidating the myogenic capacity in which myostatin operates in RBT provided additional insight into muscle development and growth along disparate evolutionary pathways, and enabled evaluation of the conservation of myogenic regulation between salmonids and mammals. While the physiology and regulation of myostatin in teleosts represent evolutionary precursors to those in mammals, the function of myostatin, as well as TGF- β molecules, appears vastly divergent in the two taxa with respect to myogenic processes. The pronounced muscularity found in myostatin-deficient mammals was not observed in teleosts in which the same ligands had been disrupted, indicating that myostatin and other TGF- β ligands do not restrict skeletal muscle development in fish; nor was exogenous somatotropin able to render a combinatorial effect alongside myostatin inhibition. While myostatin-specific inhibition produced a phenotype barely indistinguishable from that of NTG siblings, broader TGF- β interference resulted in a variant body conformation. Specific members of the TGF- β protein family, likely other than myostatin, that signal by means of the activin type IIB receptor are crucially important to the development of axial morphology, potentially influencing somite development. These proteins appear to operate in endocrine and paracrine fashions beginning early in development and persisting throughout ontogeny. They additionally function in at least a moderate capacity to constrain myocyte hyperplasia in the epaxial

musculature, which myostatin does not. Though myostatin and the related TGF- β s inhibited here are not involved in the regulation of growth rates, they do direct the potential for growth. Myostatin appears minimally, if at all, requisite for achieving growth potential, while other TGF- β ligands may restrict the capacity for growth to a greater extent. The expression of certain myogenic regulatory genes is influenced by TGF- β molecules, including myostatin, though this regulatory control did not increase myogenesis. The evolutionary preservation of the peptides inhibited here suggests that their physiological functionality has been retained, though is either not antagonistic to myogenesis or is not muscle-specific. The disparity in myostatin biology between fish and mammals would be expected to occur as a function of their respective physiologies. Mammalian evolution necessitated adaptations including heterothermy, radically different locomotory means, sweeping alterations to the proportionality and biochemistry of muscle phenotypes, and a unique mode of growth collectively brought about by novel and distinct selective pressures. A reduction in genome size may have additionally wrought the specialization of genes which once had a greater array of functions. Conversely, the somatotrophic axis remained profoundly conserved amid simultaneous modifications to the myogenic pathways. Understanding the mechanisms of processes such as vertebrate myogenesis, and the manner in which they vary across taxa, provides insight into important biological regulation and are instructive of the diversity of physiologies that may arise by means of differing evolutionary trajectories and adaptations.

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Table 1 PCR primers used for screening of transgenic F₁ individuals from ActRIIB and PD families

ActRIIB fwd	MYLC5	5'-CACCAGTCTCTTCCAAGTGTCA-3'
Pro-domain fwd	MYLC5	5'-CACCAGTCTCTTCCAAGTGTCA-3'
ActRIIB rev	ActR6	5'-TGTCCGGCAGGTGTGTAAATCTCT-3'
PD rev	MyoInt6	5'-AGAGCTGACTCCTGCATTACGTC-3'

Table 2 PCR primers used to produce *MyoD* and *myogenin* amplicons, and for sequencing clones

<i>MyoD</i> fwd	MyoD Fwd 8	5'-CTGGACTCTAGACTCGTTCATG-3'
<i>MyoD</i> rev	MyoD Rev 9	5'-ACACTTCCTGCAGGTTGTTG-3'
<i>Myogenin</i> fwd	Myogen Fwd 2	5'-CTACGATCTAGAGGACAATTCTAC-3'
<i>Myogenin</i> rev	Myogen Rev 2	5'-ACATCACCTGCAGGGACATCTAC-3'

Primers MyoD Fwd 8 & Myogen Fwd 2 contain the XbaI restriction enzyme recognition site within their sequences. Primers MyoD Rev 9 & Myogen Rev 2 contain the SbfI restriction enzyme recognition site within their sequences

Table 3 RT-qPCR primers used for all assays measuring transcriptional activity

ActRIIB qPCR fwd	Act F230	5'-GCTGGCTCGACGACTTCAACT-3'
ActRIIB qPCR rev	Act R4	5'-CATAGGGTGGCTTGTGGTGGCG-3'
<i>Myostatin</i> PD qPCR fwd	Myo F916	5'-ACGTGAATGCAGGAGTCAGCTCTT-3'
<i>Myostatin</i> PD qPCR rev	Myo R916	5'-AGGTAACCGCCAGATCATTTCCCT-3'
<i>MyoD</i> qPCR fwd	MyoD Fwd 4	5'-ACACTGAAGAGATGTACGTCTAC-3'
<i>MyoD</i> qPCR rev	MyoD Rev 4	5'-TAGTGATCCATCACCGGGTAATAG-3'
<i>Myogenin</i> qPCR fwd	Myogen Fwd 1	5'-AAGCTGTGCAAACGCAAGAC-3'
<i>Myogenin</i> qPCR rev	Myogen Rev 3	5'-TTCATCAGGGTGCTCCTCTTC-3'
<i>GAPDH</i> qPCR fwd	GAPDH Fwd	5'-ACCACTACAACCCAATCAACAGCAA-3'
<i>GAPDH</i> qPCR rev	GAPDH Rev	5'-TCGATGAAGGGATCGTTGATGGC-3'

Figure 1. Fork lengths of fish in the ActRIIB family at each of the time points. Comparisons were made on the basis of genotype and hormone treatment at each time. * indicates a significant difference between treatment groups within genotype; ** indicates a significant difference between genotypes within treatment type.

Figure 2. Mass of fish in the ActRIIB family at each of the time points. Comparisons were made on the basis of genotype and hormone treatment at each time. * indicates a significant difference between treatment groups within genotype; ** indicates a significant difference between genotypes within treatment type.

Figure 3. Condition factor of ActRIIB fish at each of the time points. Comparisons were made on the basis of genotype and hormone treatment at each time. * indicates a significant difference between treatment groups within genotype; ** indicates a significant difference between genotypes within treatment type.

Figure 4. Fork length of fish in the PD family at each of the time points. Comparisons were made on the basis of genotype and hormone treatment at each time. * indicates a significant difference between treatment groups within genotype.

Figure 5. Mass of fish in the PD family at each of the time points. Comparisons were made on the basis of genotype and hormone treatment at each time. * indicates a significant difference between treatment groups within genotype.

Figure 6. Condition factor of PD fish at each of the time points. Comparisons were made on the basis of genotype and hormone treatment at each time. * indicates a significant difference between treatment groups within genotype.

Figure 7. Specific growth rate determined for fork length in the ActRIIB family in each of the intervals between successive time points. Comparisons were made on the basis of genotype and hormone treatment within each interval. * indicates a significant difference between treatment groups within genotype.

Figure 8. Specific growth rate determined for mass in the ActRIIB family in each of the intervals between successive time points. Comparisons were made on the basis of genotype and hormone treatment within each interval. * indicates a significant difference between treatment groups within genotype.

Figure 9. Specific growth rate determined for fork length in the PD family in each of the intervals between successive time points. Comparisons were made on the basis of genotype and hormone treatment within each interval. * indicates a significant difference between treatment groups within genotype.

Figure 10. Specific growth rate determined for mass in the PD family in each of the intervals between successive time points. Comparisons were made on the basis of genotype and hormone treatment within each interval. * indicates a significant difference between treatment groups within genotype.

Figure 11. Myofiber number in ActRIIB family. * indicates a significant difference between treatment groups within genotype; ** indicates a significant difference between genotypes within treatment type; *** indicates a significant interaction between genotype and hormone treatment.

Figure 12. Myofiber diameter (μM) in the ActRIIB family. * indicates a significant difference between treatment groups within genotype; ** indicates a significant difference between genotypes within treatment type; *** indicates a significant interaction between genotype and hormone treatment.

Figure 13. Myofiber number in the PD family. * indicates a significant difference between treatment groups within genotype; ** indicates a significant difference between genotypes within treatment type; *** indicates a significant interaction between genotype and hormone treatment.

Figure 14. Myofiber diameter (μM) in fish in the PD family. * indicates a significant difference between treatment groups within genotype; ** indicates a significant difference between genotypes within treatment type; *** indicates a significant interaction between genotype and hormone treatment.

Figure 15. Expression of ActRIIB transgene construct in TG ActRIIB fish. Construct expression was normalized to *gapdh*. Expression was not significantly different between treatments. NTG fish are not represented as they do not contain the ActRIIB transgene construct.

Figure 16. *Myostatin* pro-domain expression in the ActRIIB family normalized to *gapdh*. * indicates a significant difference between treatment groups within genotype; ** indicates a significant difference between genotypes within treatment type.

Figure 17. *MyoD* expression normalized to *gapdh* in the ActRIIB fish. * indicates a significant difference between treatment groups within genotype; ** indicates a significant difference between genotypes within treatment type.

Figure 18. Expression of *myogenin*, normalized to *gapdh*, in the ActRIIB family. None of the groups differed significantly from one another.

Figure 19. Expression of the *myostatin* pro-domain normalized to *gapdh* in the PD family. * indicates a significant difference between treatment groups within genotype; ** indicates a significant difference between genotypes within treatment type.

Figure 20. *MyoD* expression normalized to *gapdh* in the PD family. * indicates a significant difference between treatment groups within genotype; ** indicates a significant difference between genotypes within treatment type.

Figure 21. Expression of *myogenin* in the PD family normalized to *gapdh*. * indicates a significant difference between treatment groups within genotype; ** indicates a significant difference between genotypes within treatment type.

APPENDIX A: CORRELATION ANALYSES

The data and results contained herein were collected from the original study and are intended to serve as supplemental information within the context of the findings presented in the thesis.

The expression and histological data were utilized in correlation analyses to glean putative relationships between the expression of the various genes of interest, as well as some that may have existed between the expression of such genes and the mitotic and/or growth physiology of the epaxial myocytes. Correlations were carried out between the expression of *MyoD* and *myostatin*, *myogenin* and *myostatin*, and *MyoD* and *myogenin* in both the ActRIIB and PD families. Additionally, in the ActRIIB family, correlations between the ActRIIB construct and *myostatin* expression levels were performed; this could only be done for the TG fish as the NTG individuals lacked the construct entirely. In both families *myostatin* expression was regarded as indicative of levels of the transcript which encodes the myostatin pro-peptide in which the pro-domain and bioactive portion remain bound prior to post-translational processing.

Along with correlations relating expression levels were those comparing myofiber number and diameter for both families. Myofiber number and the expression of *MyoD* were correlated for all experimental groups in the ActRIIB and PD families. The number of samples available for comparisons ($N = 10$ for gene expression correlations, $N = 6$ for histological correlations) instructed the use of both Pearson and Spearman correlations to account for relationships that had the potential to be obscured by the nature of the data sets. Those correlations reported as significant, however, are only noted as such in those instances in which the test most appropriate to the distribution of the data set revealed a significant coefficient.

Relatively few of the correlations analyzed in either of the families proved significant. In both the rbST-treated and placebo ActRIIB TG fish there was a positive relationship between the expression of the ActRIIB construct and *myostatin* which was not significant (Fig. 22). The coefficients for comparisons of *MyoD* and *myostatin* expression ranged from weak to moderate, none of which achieved significance (Fig. 23). The coefficients comparing *myogenin* and *myostatin* for all groups in the ActRIIB family indicated fairly weak correlations, none of which were significant (Fig. 24). Despite fluctuations in magnitude of the correlations between the various groups, none of the comparisons relating *MyoD* and *myogenin* expression proved significant in the ActRIIB family (Fig. 25).

The relationship between the number of myofibers and their diameters in the ActRIIB fish was defined in all groups by an inverse relationship in which individuals with a greater number of myofibers were those with smaller diameter fibers., Correlations between diameter and number were significant in placebo TG fish ($r^2 = -0.90$) and the rbST-treated NTG fish ($r^2 = -0.89$) (Fig. 26). Myofiber number and *MyoD* expression, however, were not found to be significantly correlated in these fish (Fig. 27).

In the PD fish *MyoD* expression was significantly related to that of *myostatin* in the placebo TG fish ($r_s^2 = 0.77$) and the placebo NTG fish ($r^2 = 0.75$), with an increase in expression of *MyoD* corresponding to increased expression of *myostatin* (Fig. 28). *Myogenin* expression increased significantly in accord with *myostatin* in the rbST-treated TG fish ($r^2 = 0.67$); however, this significance was exclusive to this group in the PD fish (Fig. 29). As in the ActRIIB fish, the relationship between *MyoD* and *myogenin* transcription in the PD fish was not significant in any of the fish (Fig. 30).

The TG rbST-treated and placebo fish exhibited a significant inverse relationship ($r^2 = -0.84$ and $r^2 = -0.98$, respectively) between myocyte number and diameter, i.e., increased myofiber diameters were found in fish with fewer numbers of fibers (Fig. 31). *MyoD* expression was not significantly correlated with myocyte number in the PD family, similar to what had been observed in the ActRIIB fish (Fig. 32).

Table 4 Pearson (r^2) and Spearman (r_s^2) correlations for parameters in the ActRIIB family. * indicates a significant correlation; correlations are denoted as significant for those tests which were most appropriate to the data set.

Correlation	Group	r^2	p	r_s^2	p
ActRIIB-Myostatin (PD) expression	rbST-treated TG	0.47	0.17	0.54	0.11
	placebo-treated TG	0.21	0.56	0.18	0.63
MyoD-Myostatin (PD) expression	rbST-treated TG	-0.47	0.17	-0.47	0.17
	placebo-treated TG	0.35	0.33	0.42	0.23
	rbST-treated NTG	0.63	0.05	0.59	0.07
	placebo-treated NTG	0.37	0.29	0.25	0.49
Myogenin-Myostatin (PD) expression	rbST-treated TG	0.35	0.32	0.21	0.56
	placebo-treated TG	0.28	0.43	0.26	0.47
	rbST-treated NTG	-0.02	0.95	-0.13	0.73
	placebo-treated NTG	0.5	0.14	0.22	0.53
MyoD-Myogenin expression	rbST-treated TG	-0.50	0.14	-0.59	0.07
	placebo-treated TG	0.55	0.10	0.59	0.07
	rbST-treated NTG	0.36	0.31	0.25	0.49
	placebo-treated NTG	0.79	0.01	0.47	0.17
Myofiber number-MyoD expression	rbST-treated TG	-0.26	0.62	-0.03	0.96
	placebo-treated TG	-0.08	0.86	0.14	0.79
	rbST-treated NTG	0.19	0.72	0.43	0.40
	placebo-treated NTG	-0.75	0.08	-0.14	0.79
Myofiber number-Myofiber diameter	rbST-treated TG	-0.55	0.26	-0.43	0.40
	placebo-treated TG	-0.9	0.01*	-0.49	0.33
	rbST-treated NTG	-0.89	0.02*	-0.77	0.07
	placebo-treated NTG	-0.33	0.52	-0.54	0.27

Table 5 Pearson (r^2) and Spearman (r_s^2) correlations for parameters in the PD family.
 * indicates a significant correlation; correlations are denoted as significant for those tests which were most appropriate to the data set.

Correlation	Group	r^2	p	r_s^2	p
<i>MyoD-Myostatin</i> (PD) expression	rbST-treated TG	0.29	0.42	0.3	0.40
	placebo-treated TG	0.35	0.32	0.77	0.01*
	rbST-treated NTG	0.36	0.30	0.41	0.24
	placebo-treated NTG	0.75	0.01*	0.62	0.05
<i>Myogenin-Myostatin</i> (PD) expression	rbST-treated TG	0.67	0.03*	0.61	0.06
	placebo-treated TG	0.4	0.26	0.35	0.33
	rbST-treated NTG	0.04	0.90	0.25	0.49
	placebo-treated NTG	0.06	0.88	0.21	0.56
<i>MyoD-Myogenin</i> expression	rbST-treated TG	-0.12	0.75	0.04	0.91
	placebo-treated TG	0.05	0.88	0.04	0.91
	rbST-treated NTG	0.22	0.54	0.36	0.31
	placebo-treated NTG	0.06	0.88	0.05	0.88
Myofiber number- <i>MyoD</i> expression	rbST-treated TG	0.53	0.27	0.49	0.33
	placebo-treated TG	0.09	0.87	0.26	0.62
	rbST-treated NTG	-0.16	0.76	-0.14	0.79
	placebo-treated NTG	-0.41	0.43	-0.20	0.70
Myofiber number-Myofiber diameter	rbST-treated TG	-0.84	0.04*	-0.60	0.21
	placebo-treated TG	-0.98	<0.00	-0.89	0.02*
	rbST-treated NTG	0.19	0.71	0.09	0.87
	placebo-treated NTG	-0.80	0.05	-0.60	0.21

Figure 22. Expression of *myostatin* and ActRIIB transgene construct in rbST-treated and placebo TG ActRIIB fish. Neither treatment group exhibited a significant correlation between expression of the two genes.

Figure 23. *MyoD* and *myostatin* pro-domain expression in the ActRIIB family. Correlations were not significant in any of the groups.

Figure 24. Expression of *myostatin* pro-domain and *myogenin* in the ActRIIB family. There were no significant relationships in the expression of the two genes in any of the groups.

Figure 25. Expression of *MyoD* and *myogenin* in the ActRIIB family. None of the groups exhibited a significant relationship between the transcription of the two genes.

Figure 26. Myofiber number and diameter in the ActRIIB family. In all groups increasing numbers of fibers were correlated with a smaller myofiber diameter. The relationship between number and diameter was significant in the placebo TG fish and NTG rbST-treated fish.

Figure 27. Myofiber number and *MyoD* expression in the ActRIIB family. None of the groups exhibited a significant relationship between gene expression and myofiber number.

Figure 28. *MyoD* and *myostatin* transcription levels in the PD family. Transcription of the two genes was significantly correlated in the placebo fish of both genotypes, though not in the rbST-treated fish.

Figure 29. *Myogenin* and *myostatin* expression in the PD family. Expression of the two genes was significantly correlated in only the TG rbST-treated fish.

Figure 30. *MyoD* and *Myogenin* expression in the PD fish. The relationship between the two genes was not significant in any of the groups.

Figure 31. Myofiber number and diameter in the PD family. Fiber number was significantly related to fiber diameter in all TG fish.

Figure 32. Myofiber number and *MyoD* expression in the PD family. Gene expression was not significantly correlated to myofiber number in any of the groups.

APPENDIX B: LITERATURE REVIEW

Myostatin

The transforming growth factor- β (TGF- β) cytokines comprise a superfamily of closely related endocrine factors which are involved in the regulation of an array of cellular processes which mediate growth processes in vertebrates. Despite a number of shared characteristics in their structure and maturation, the peptides within this substantial group of related proteins instruct physiological processes in a suite of different cell types and tissues. They function by binding to an assortment of cellular receptors, and initiate intracellular cascades mediated by a still greater number of signaling molecules and transcription factors; they are also subject to regulation themselves in a number of capacities by a variety of mechanisms. One of the members of the TGF- β superfamily, myostatin, has been prolifically and intensively studied as it serves to negatively regulate skeletal muscle growth in mammals (McPherron et al., 1997; Rodgers and Garikipati, 2008). Myostatin has monopolized research efforts as it has implications in the biomedical, agricultural, regenerative medicine, and pathology fields (Joulia-Ekaza and Cabello, 2006; Rodgers and Garikipati, 2008; Lee, 2004).

Elucidation of the structure of the myostatin proteins in bovine species revealed that they contained a number of the characteristics which distinguish, and are shared by, TGF- β ligands (McPherron and Lee, 1997; McPherron et al., 1997). Myostatin, similar to other TGF- β ligands, contains a secretion-directing domain, a conserved RSRR sequence directing proteolytic cleavage and processing, and a highly conserved carboxy-terminal domain which is defined by the presence of a number of cysteine residues (McPherron et al., 1997; de Caestecker, 2004). The unwavering patterning of these cysteine residues is crucial to the ability of myostatin to assume a functional

conformation and forms a cysteine knot through disulfide bonds joining these residues (McPherron et al., 1997; McPherron and Lee, 1997).

Myostatin, unlike some of the related TGF- β s, is initially synthesized as an inactive precursor in which the biologically active C-terminal domain is bound to the amino-terminal pro-domain portion of the peptide (sometimes referred to as a latency associated protein, LAP) as well as other proteins, including the latent TGF- β binding protein (LTBP) in a latent complex (Lee and McPherron, 2001; de Caestecker, 2004; Thomas et al., 2000; Munger et al., 1997). TGF- β ligands retained within this latent complex appear to be bound to the extracellular matrix following secretion from the cell, and proteolytic or non-proteolytic cleavage by factors such as plasmin or thrombospondin, respectively, may free the ligand, at which point it would remain complexed with the pro-domain region (Munger et al., 1997; Lee and McPherron, 2001). Myostatin appears, even in its inactive form, to dimerize by means of disulfide linkages, and subsequent proteolytic cleavage at the RSRR sequence site by BMP-1 (bone morphogenetic protein)/tolloid metalloproteinase family members releases the C-terminal homodimer active peptide from the N-terminal pro-domain, at which point the 376 residue C-terminal active domain is rendered physiologically viable (McPherron et al., 1997; Thomas et al., 2000; Lee and McPherron, 2001; Lee, 2004). The functionality of myostatin relies on its release from the latency complex as well as the N-terminal pro-domain (Lee and McPherron, 2001); Thomas et al. (2000) established that the latency complex-associated form of myostatin, as well as the inactive, pro-domain-bound form are contained within myoblasts, while the smaller, mature myostatin protein is not. The mature protein was instead located in the skeletal muscle, indicating it was secreted

following release from the pro-domain, and likely defining myoblasts as the site of proteolytic processing (Thomas et al., 2000).

Myostatin exerts its regulatory control of mammalian skeletal muscle through signaling cascades and mechanisms employed by most, if not all, TGF- β ligands (Kemaladewi et al., 2011; Lee, 2004; de Caestecker, 2004; Lee, 2007; Derynck and Zhang, 2003). There are two, highly similar, receptor classes (type I and type II) which bind circulating myostatin as well as other TGF- β ligands (Lee, 2007; de Caestecker, 2004; Derynck and Zhang, 2003). The extracellular binding domain of both receptor types is defined by a three-finger toxin fold conformation, and both types possess both a single membrane-spanning domain and intracellular serine-threonine kinase domain (Derynck and Zhang, 2003; de Caestecker, 2004). The type I and type II receptor classes differ with respect to the sequence of their kinase domains, as well as the existence of a defined glycine-serine sequence (commonly referred to as the GS box) found on the type I receptors but absent in the type II receptors; this GS box remains crucial for type I receptor activation (de Caestecker, 2004; Massagué, 2000).

The two classes of TGF- β receptors commonly exist in homodimeric associations within the cell membrane when not bound by ligands; however, myostatin binding of the type II receptor inspires association with the type I receptor (also referred to as the Activin-like kinase, ALK) (Derynck and Zhang, 2003). Myostatin appears to preferentially bind the type II receptor initially, specifically the Activin type IIB receptor as opposed to the type IIA receptor (Lee and McPherron, 2001; Lee, 2007; Derynck and Zhang, 2003). As binding of the C-terminal active myostatin domain induces association with the type I receptor (ALK4 in myocytes, Kemaladewi et al., 2011) a heteromeric

receptor tetramer complex is formed, and the intracellular kinase domain of the type II receptor transphosphorylates the GS box of the type I receptor, activating it (Lee, 2007; Massagué, 2000). The activated GS domain subsequently phosphorylates intracellular receptor Smad (R-Smad) proteins, in the case of myostatin signaling Smad2 and Smad3 are phosphorylated and subsequently associate with a coeffector, Smad4 (Langley et al., 2002; Liu et al., 2001; Derynck and Zhang, 2003; Ge et al., 2011). The activated Smads translocate to the nucleus where they suppress transcription of myogenic genes including *MyoD* and *Pax-3*, functionally interacting with and inhibiting the actions of myogenic transcription factors such as MyoD, and precluding muscle growth (Liu et al., 2001; Derynck and Zhang, 2003; Amthor et al., 2004).

The regulation of myogenesis by myostatin is a function of the peptide's disruption of cell cycle progression, as well as functionally mediating myoblast and satellite cell populations, and differentiated muscle tissue, by means of governing transcriptional activity and mitogenic pathways (Thomas et al., 2000; Joulia-Ekaza and Cabello, 2006; Wang and McPherron, 2012; Amthor et al., 2009; Taylor et al., 2001; Langley et al., 2002; McCroskery et al., 2003; Garikipati and Rodgers, 2012; Liu et al., 2001). Myostatin was demonstrated to inhibit myocyte proliferation *in vitro*, an effect which could be reversed with cessation of myostatin administration to the cell cultures (Thomas et al., 2000; Talyor et al., 2001). Regulation of the cell cycle was found to be mediated by the expression of genes which serve as mitotic effectors, which were influenced by myostatin signaling (Thomas et al., 2000). Advancement of cells from the G₁ to the S phase during mitosis is governed in large part by the enzymatic activities of cyclin-dependent kinases (CDKs) complexed with specific cyclins, the actions of which

are in turn modulated by CDK inhibitors such as p21 (Thomas et al., 2000). The role of CDKs in the cell cycle remains essential as they phosphorylate the retinoblastoma (Rb) protein, thereby inactivating it, as the Rb protein in its hypophosphorylated form halts mitotic progression (Thomas et al., 2000). Notably, Thomas et al. (2000) found that administering myostatin to cell cultures concomitantly upregulated p21 and reduced both the activity and concentration of cyclin E-CDK2 complexes. Moreover, myostatin was found to upregulate p21 solely, rather than any of the other CDK inhibitors, and increase concentrations of hypophosphorylated Rb protein (Thomas et al., 2000).

Myostatin, therefore, was suggested to inhibit muscle growth at least in part through its regulation of myoblast proliferation (Thomas et al., 2000). These investigators additionally introduced myostatin to myocyte cultures derived from wild type and Belgian Blue cattle, which exhibit dramatically increased skeletal muscle, and demonstrated that cells from both cattle breeds were inhibited by myostatin. These data confirm that in the Belgian Blue cattle the increased muscling is the result of a nonfunctional myostatin protein rather than a receptor, as deregulation of the receptor would preclude growth inhibition of Belgian Blue cell cultures by myostatin.

Myostatin, in addition to disrupting the mitotic progression of myoblasts, was found to constrain protein synthesis in C₂C₁₂ cell cultures (Taylor et al., 2001). These effects were not attributable to apoptotic events, though the reduction in protein aggregation may have been secondary to reduced muscle accretion generally as a result of myostatin's actions (Taylor et al., 2001). Antagonism of muscle growth by myostatin has additionally been found to result from perturbation of cellular differentiation (Langley et al., 2002; Liu et al., 2001). Transcriptional repression by myostatin was

demonstrated to be mediated by Smad3, which is able to intrude upon the functionality of the transcription factor MyoD by means of direct interaction with the basic Helix-Loop-Helix (bHLH) motif of the protein (Liu et al., 2001; Langley et al., 2002). Down regulation of genes specifying the myogenic lineage, such as *myogenin*, and disruption of MyoD activity and expression, effectively hinder the pathways leading to lineage specification and differentiation (Langley et al., 2002; Liu et al., 2001).

Muscle satellite cells, myogenic stems cells which may be recruited in the event of post-natal muscle repair, and perhaps for growth, are also subject to regulation by myostatin (McCroskery et al., 2003; Wang and McPherron, 2012). While myostatin was demonstrated to negatively influence replenishment of satellite cell populations, and suppress activation of these cells (McCroskery et al., 2003) it may be that muscle growth relies minimally, or not at all, on recruitment of these cells to increase tissue mass (Wang and McPherron, 2012; Amthor et al., 2009). Muscle growth was found to result not from hyperplasia subsequent to satellite cell activation, but rather from hypertrophy of existing myofibers (Wang and McPherron, 2012; Amthor et al., 2009). Only minimal satellite cell activation was found in myostatin-inhibited adult mice, and the hypertrophic growth that took place occurred predominantly within existing myofibers, without the recruitment of latent cells; thus indicating that myostatin acts more extensively on fully differentiated tissue (Wang and McPherron, 2012).

While these data appear to conflict with those concerning the inhibition of myoblast proliferation by myostatin the seemingly disparate mechanisms of myostatin regulation may not be entirely mutually exclusive. It may be that myostatin functions in distinct capacities to regulate muscle development at various points throughout ontogeny.

Myostatin may operate in a temporally delimited fashion throughout the life history of the organism; wherein the mechanisms employed for the regulation of muscle growth in embryonic, or early post-natal development, may differ from those that regulate growth later. Conceivably, restraining proliferation earlier in development to limit muscle growth may give way to the inhibition of protein synthesis in fully differentiated tissue; alternatively, there may exist some overlap or synchronicity of these events.

Physiological regulation of myostatin is both dynamically structured and versatile, occurring by means of numerous mechanisms and thereby enabling precise control of its signaling and actions. Myostatin is beholden to both intracellular and extracellular regulatory effectors, which may act on the molecule specifically, or rather as only one of a multitude of ligands moderated by a particular entity. Additionally, the regulation of myostatin may occur along the signaling pathway, while present in circulation, or at the transcriptional level. Decorin, a proteoglycan which associates with the collagen fibrils of the extracellular matrix, and which is known to regulate TGF- β ligands, is profoundly capable of regulating myostatin and its actions (Miura et al., 2006; Kishioka et al., 2007; Zhang et al., 2012). Proliferation of C₂C₁₂ cells overexpressing decorin exhibited increased proliferation rates and accelerated progression through the cell cycle, as well as improved rates of differentiation (Kishioka et al., 2007; Miura et al., 2006). Moreover, myostatin binding by decorin appears to interfere with an autoendocrine inhibitory feedback mechanism employed by myostatin in which the protein binds the gene promoter to modify its expression (Kishioka et al., 2007).

Myostatin-null mice were found to have increased decorin expression, which led to contrasting effects on healing within different tissues following induced wounding

(Zhang et al., 2012; McCroskery et al., 2005). While satellite cell activation and migration to the wound site was increased, and muscle repair was augmented by enhanced macrophage enlistment and consequently improved inflammatory response in these mice, healing of the dermal and epidermal layers was antagonized in the absence of myostatin (McCroskery et al., 2005; Zhang et al., 2012). This was attributed to the up-regulation of decorin, which subsequently disrupted the actions of other TGF- β s in the skin which are required for repair, such as those directing keratinocytes; which collectively point to disparate tissue-specific roles for myostatin (Zhang et al., 2012).

Myostatin inhibition can additionally be accomplished by the pro-domain of the endogenous peptide, as well as other endocrine factors, and receptor regulation. When bound to the N-terminal pro-domain portion of the peptide, the activity of the C-terminal active domain is suppressed and it is only following cleavage from the pro-domain that the mature molecule can become active (Lee and McPherron, 2001). The pro-domain is therefore able to quell myostatin levels, serving as a primary control mechanism of the concentration of the physiologically active ligand. An array of other molecules bind myostatin in circulation, or locally within the muscle tissue, some of which bind the mature peptide and others of which bind the latent form; some factors may bind both forms (Joulia-Ekaza and Cabello, 2006). One such molecule, follistatin, effectively binds to and represses myostatin activity in both mammalian and non-mammalian species (Haidet et al., 2008; Rebhan and Funkenstein, 2008; Medeiros et al., 2009; Lee et al., 2010). The complexity and flexibility of myostatin binding is further elaborated by molecules such as growth and differentiation factor-associated serum protein-1 (GASP-1), follistatin-related gene (FLRG), titin-cap, and human small glutamine-rich

tetratricopeptide repeat-containing protein (hSGT), which are able to variously bind mature and/or immature myostatin in circulation and/or within the skeletal muscle (Joulia-Ekaza and Cabello, 2006; Wang et al., 2003; Hill et al., 2002; Hill et al., 2003). As myostatin antagonists these factors are fundamental to maintaining proper skeletal muscle growth and homeostasis.

The mode of TGF- β receptor endocytosis has also been established as a means of regulating the viability of TGF- β ligands (Di Guglielmo et al., 2003), myostatin presumably among them. Cell surface TGF- β receptors internalized within clathrin-coated pits are maintained and their signals faithfully transduced to intracellular receptors (Di Guglielmo et al., 2003). Alternatively, if the receptors are endocytosed by means of caveolin-coated caveolae the ligand-bound receptor complex is slated for degradation and the signal fails to be transduced (Di Guglielmo et al., 2003). Intracellular regulation of myostatin signaling may also be mediated by Smad proteins, as the translocation of the R-Smads to the nucleus is subject to independent regulation, and inhibitory effectors such as Smad6 and Smad7 target signaling R-Smads for degradation (Derynck and Zhang, 2003). Transcriptional and post-translational regulation of myostatin in some teleost species may also be affected through somatotropin expression (Roberts et al., 2004; Roberts and Goetz, 2003). In brook trout (*Salvelinus fontinalis*) the 5' regulatory regions in both *myostatin* alleles were found to contain a somatostatin-responsive sequence domain, suggesting regulation by an anabolic factor at the genomic level (Roberts and Goetz, 2003).

Myostatin functions to regulate somatic growth as a negative endocrine effector of skeletal muscle growth in mammals (McPherron and Lee, 1997; Kambadur et al.,

1997). Several mammalian species possessing mutations in the myostatin gene exhibit a dramatic muscular phenotype, commonly referred to as double-muscled breeds (Clop et al., 2006; Mosher et al., 2007; McPherron and Lee, 1997). The increased muscling in domesticated breeds such as Texel sheep, Piedmontese and Belgian Blue cattle, and “bully” whippets has been attributed to nonfunctional myostatin in the skeletal muscle (Clop et al., 2006; Mosher et al., 2007; Kambadur et al., 1997; McPherron and Lee, 1997). Texel sheep were found to possess a mutation in the 3' untranslated region (3' UTR) of the myostatin gene that gave rise to a site which could be exploited by microRNAs, leading to transcript degradation and failed signaling (Clop et al., 2006). The phenotype observed in the Belgian Blue cattle was found to result from a frameshift in the third exon due to an 11-nucleotide deletion, which led to truncation of the protein and the absence of nearly the complete active portion; by contrast the Piedmontese phenotype is caused by the loss of one of the cysteine residues essential to the formation of the cysteine knot in the C-terminal (McPherron and Lee, 1997; Kambadur et al., 1997). Myostatin deregulation attributable to a mutant allele and leading to muscle enhancement has been discovered in humans as well (Schuelke et al., 2004).

The physiological administration of myogenic processes by myostatin that have been observed in domesticated mammals, and the phenotypes generated by its deregulation, have become better understood through experimental manipulation of the peptide. *Myostatin*-null mice exhibit significantly greater skeletal muscle mass, and a phenotype reminiscent of the double-muscling seen in myostatin-deficient cattle (McPherron and Lee, 1997; McPherron et al., 1997; Yang et al., 2001; Zhu et al., 2000; Lee, 2007; Thies et al., 2001; Lee and McPherron, 2001). Transgenic mice expressing

the myostatin antagonist follistatin, as well as those expressing a dominant negative isoform of the Activin IIB receptor (ActRIIB), part of the heterodimeric receptor complex through which myostatin signals, exhibited increased muscle mass due to both hyperplastic and hypertrophic skeletal muscle growth (Lee and McPherron, 2001). Zhu et al. (2000) generated transgenic mice expressing a dominant negative isoform of the endogenous myostatin protein, though discovered that the muscle growth observed was the result of hypertrophy rather than hyperplasia; they suggested that the level or method of myostatin knockdown in this approach may account for the difference in the mode of muscle enhancement.

Increased myogenesis was also achieved in transgenic models using follistatin and the pro-domain region of the endogenous myostatin peptide (Yang et al., 2001; Thies et al., 2001; Lee et al., 2010; Lee, 2007). Transgenic expression of the myostatin pro-domain in a murine model led to increased hypertrophic growth in a manner proportional to the level of construct expression, though it was acknowledged that the increases were more moderate compared to those observed in myostatin knockout mice (Yang et al., 2001). Thies et al. (2001) demonstrated the specificity of the myostatin pro-domain in its actions on myostatin, suggesting that despite the presence of a pro-domain region in other TGF- β ligands the crosstalk between these inhibitory N-terminal domains and the various ligands of the superfamily may be minimal or nonexistent. Furthermore, they observed a differential regulation by myostatin of the factors involved in glycolytic and oxidative fiber development; factors involved in glycolytic fiber development such as MyoD were up-regulated in myostatin null mice, while those involved in oxidative fiber growth were conversely suppressed.

In vivo analysis found myostatin complexed with the pro-domain in circulation, while FLRG was similarly able to inhibit myostatin activity (Hill et al., 2002). In the chick, myostatin down-regulated myogenic factors such as *Myf-5* and *Pax-3* embryonically (Amthor et al., 2002). Follistatin effectively bound myostatin, halting down-regulation of these myogenic genes, and colocalized with myostatin to some extent during embryonic limb bud development in the chick; suggesting that these two contrasting signaling effectors perhaps work somewhat cooperatively, if in opposition, to structure muscle tissue embryonically (Amthor et al., 2004).

While myostatin has come to be understood as indispensable in mammalian myogenesis it does not appear to function unilaterally. Regulatory molecules such as follistatin are able to bind not only myostatin, but also other TGF- β ligands; making it a broadly competent effector capable of potentially binding other myogenic antagonists (Lee et al., 2010). It has also been suggested that Activin A, another TGF- β family member, may work in addition to myostatin to regulate muscle development (Lee et al., 2010). To this end, muscle growth exceeding that found in *myostatin*-null mice was accomplished as TGF- β ligands were simultaneously inhibited alongside myostatin (Lee, 2007; Lee et al., 2005). *Myostatin*-null mice bred with mice overexpressing follistatin were able to achieve greater muscle mass than either of the singly transgenic individuals (Lee, 2007). Similarly, the introduction of a soluble ActRIIB receptor against a *myostatin*-null genetic background generated muscle mass beyond *myostatin* knockout independently (Lee et al., 2005). These data collectively point to the involvement of other TGF- β , or even unrelated, ligands in mediating the physiology of mammalian muscle growth.

The characterization and understanding of myostatin function in mammals does not extend into other phylogenetic groups. Myostatin has been discovered in a number of teleost species, in many cases multiple gene copies have been determined and their tissue-specific expression resolved (Roberts and Goetz, 2001; Rodgers et al., 2001; Østbye et al., 2007; Radaelli et al., 2003; Rescan et al., 2001). Unlike mammals, in which myostatin is expressed in skeletal muscle tissue and very few others, teleosts express myostatin in a wide variety of tissues (Rescan et al., 2001; Radaelli et al., 2003; Roberts and Goetz, 2001). Differential expression of the isoforms in the tissues is observed in a number of species; the expression of *myostatin1* was ubiquitous in the tissues examined, while *myostatin2* expression was limited to the brain and skeletal muscle (Radaelli et al., 2003; Rescan et al., 2001; Roberts and Goetz, 2001). The expression profiles of the two genes were found to differ between oxidative and glycolytic muscle tissue as well (Roberts and Goetz, 2001).

Attempts to resolve the precise physiological function of myostatin in fish have been frustrated by the presence of multiple isoforms and expression profiles which deviate widely from those in mammals, characteristics owing to the evolutionary distance between these lineages. Some of these disparities may be accounted for by the multiple genome duplication events which occurred within teleosts over the course of their evolution, and which brought about multiple gene paralogues (Jaillon et al., 2004; Amores et al., 1998; Rescan et al., 2001). Such duplication events enable evolution to act, perhaps independently, on these distinct isoforms, wherein some may retain their ancestral function while others are able to elaborate novel utilities and transcripts. The human, rat, mouse, baboon, porcine, bovine, chicken, turkey, ovine, and zebrafish (*Danio*

rerio) predicted amino acid sequences all contain the conserved proteolytic cleavage site, and the active domain of the molecule in zebrafish shares substantial similarities (88%) with those of more derived vertebrates (McPherron and Lee, 1997; Rescan et al., 2001).

The sequence and structural conservation found in myostatin across taxa may belie a functional incongruity which exists between its physiological role in teleosts and other vertebrates. Experimental inhibition of myostatin in bony fish has led to alterations in the musculature and myogenic processes, but may not signal a substantial role for this ligand in the muscle regulation of these fish (Xu et al., 2003; Rebhan and Funkenstein, 2008; Seiliez et al., 2012; Garikipati and Rodgers, 2012; Medeiros et al., 2009; Martin and Johnston, 2005). Muscle growth in teleosts occurs in a manner distinct from that in mammals, and a number of bony fish possess an indeterminate growth potential (Rescan, 2005; Xu et al., 2003; Johnston et al., 2003). In teleosts both mosaic and stratified hyperplasia contribute to muscle growth at different points in ontogeny (Rescan, 2005; Johnston et al., 2003). The dorsal and ventral germinal regions of the developing myotome undergo a stratified hyperplasia as spatially distinct increases in fiber number occur at different points along the dorso-ventral axis of the somite (Rescan, 2005). Later in development, as well as postembryonically, this stratification gives way to hyperplastic growth occurring throughout the myotome as dispersed myogenic progenitor cells either continue proliferating to form new fibers, or augment the structure of existing fibers (Rescan, 2005; Johnston et al., 2003). In this way the architecture of the myotome is defined by a heterogeneous population of fibers (Rescan, 2005; Johnston et al., 2003).

Expression of the myostatin pro-domain in transgenic zebrafish led to minimal increases in the musculature which were attributed to stratified, but not mosaic,

hyperplasia (Xu et al., 2003). These findings corresponded with differential temporal expression of myostatin in zebrafish, which was minimal in the embryos (Xu et al., 2003). These results were found, however, in a species with determinate growth, and may not be applicable to indeterminately growing fishes. While follistatin and pro-domain constructs were both found to efficiently bind gilthead seabream (*Sparus auratus*) myostatin this was found *in vitro* and the impacts on muscle development were not elucidated (Rebhan and Funkenstein, 2008). Similarly, myostatin was found to promote differentiation in rainbow trout (*Oncorhynchus mykiss*) cells, whereas it is inhibitory to differentiation in mammalian cells (Garikipati and Rodgers, 2012). Rainbow trout overexpressing follistatin had increased muscling as a result of hyperplasia, and exhibited a distinctly muscled phenotype which may be caused by interference with myostatin, or other TGF- β ligands (Medeiros et al., 2009).

The diversity of function that exists for myostatin and TGF- β ligands may be accounted for by the extensive suite of regulatory mechanisms that may influence these endocrine factors, and may potentially underlie some of the interspecific differences in their functions. The TGF- β signaling cascade is initiated by ligand binding to the extracellular domain of the TGF- β receptors, which elicits the assembly of a receptor complex (Derynck and Zhang, 2003). However, the particular receptor components comprising the receptor complex can vary depending upon the cellular context, and can either bind multiple ligands, or initiate different signaling cascades when bound by the same ligand (de Caestecker, 2004; Derynck and Zhang, 2003; Kemaladewi et al., 2011). This diversity creates a vast number of potentials for signaling and gene expression in different tissues and at different points in development. The intracellular signaling R-

Smads may additionally associate in various conformations; the (Mad Homology) MH1 and MH2 domains of the Smad proteins are able to associate with sequence-specific transcription factors, and their C-terminal domains draw in transcriptional coactivators (Derynck and Zhang, 2003). Additionally, sequences within the receptors, and the MH2 domains of the R-Smads confer binding specificity, and R-Smad activation relies on phosphorylation of serine residues within the C-terminal SSXS sequence (Massagué, 2000). Smads are subject to independent regulation and degradation by Smurf1 and Smurf2 (Smad-ubiquitination-regulatory factor); and complexes such as SARA (Smad anchor for receptor activation) and TRAP-1 (TGF- β -receptor-associated protein) participate in R-Smad-receptor association (Derynck and Zhang, 2003). Moreover, TGF- β ligands can directly activate MAPK (mitogen activated protein kinase) pathways without Smad intermediaries (Derynck and Zhang, 2003). Master transcription factors present in different cell types were discovered to bind Smad 3 following TGF- β activation and direct the expression of cell-specific genes within these tissues, accentuating the essential nature of the cellular context in which this signaling occurs (Mullen et al., 2011). Various cell-specific cofactors were additionally found to be requisite for myostatin signaling in muscle cells (Kemaladewi et al., 2011). Both ALK4 and Cripto (also referred to as teratocarcinoma-derived growth factor1, Tdgf1) were required for effective signal transduction of myostatin in myocytes, while myostatin relied on ALK5 expression in nonmyogenic cells to signal (Kemaladew et al., 2011). These data collectively illustrate the tremendous complexity involved in TGF- β signaling, and the diversity of responses that may be elicited.

As a member of this superfamily of ligands, myostatin may differentially influence the cellular growth and differentiation of various cell types depending upon the signaling cascade initiated, or the cofactors and the program of transcriptional activation involved. These pathways may also be influenced differently throughout ontogeny, and lead to a variety of physiological responses during the life history of the organism. The array of components involved in myostatin, and TGF- β , signaling similarly provide a platform for evolution to develop intensely divergent mechanisms and functions in disparate physiological and cellular contexts.

MyoD and Myogenin

The architecture of teleost skeletal muscle remains unique among vertebrates, evolved for maneuvering through a medium far denser than that encountered by terrestrial organisms. Along the length of the body the myofibers of the skeletal muscle are arranged in serially segmented myotomes, which are delineated by the myosepta comprised of connective tissue (Jobling, 1994). The phenotypes of the fibers are also distinct, with the aerobic and anaerobic fibers more acutely delineated in most teleosts than in mammals and other vertebrates (Jobling, 1994). The anaerobic, or fast, muscle fibers comprise the majority of the muscle mass in teleosts, positioned medially within the body and occupying much of the myotome; while the aerobic muscle represents comparatively little of the skeletal muscle, positioned exterior to the anaerobic muscle immediately beneath the dermis and following the approximate contour of the lateral line (Jobling, 1994). Within the myotomes the angle of attachment of individual anaerobic muscle fibers ranges broadly, from fairly minimal to more severe; the collective result of these oblique angles is to lend a somewhat helical conformation to the musculature along

the rostral-caudal axis of the body (Jobling, 1994). By contrast, the aerobic fast fibers display minimal heterogeneity in their angle and are arranged lengthwise along the longitudinal axis of the body (Jobling, 1994). The skeletal muscle consists predominantly of anaerobic fibers, with aerobic, and in some instances fibers of intermediate phenotype and physiology, comprising relatively little of the musculature (Jobling, 1994). The relative proportions of these fiber types, however, differ between species, reflecting the ecological niche occupied by the fish, as well as the evolutionary trajectories of various clades.

In many regards myogenesis in bony fish may be considered, genetically and developmentally, indistinguishable from that in other vertebrates; indeed, it represents the basal vertebrate condition. However, the genome duplication events experienced by the salmonid lineages have led to functional diversification of muscle-specific paralogs (Delalande and Rescan, 1999; Macqueen and Johnston, 2006); and teleosts exhibit embryonic cellular migrations and patterning unique to their taxon (Stellabotte et al., 2007; Hollway et al., 2007). As in other vertebrates, discrete regions within the developing somites of teleosts adopt particular cell morphologies while the pluripotency of the cells within the somitic domain simultaneously narrows (Gilbert, 2006). The shifting morphology and gene expression of somatic locales give rise to the myoblast-producing myotome, and eventually the dermomyotome (Gilbert, 2006; Devoto et al., 2006). These somitic derivatives produce the myoblasts which eventually form the multinucleated syncytial muscle fibers; various regions within the myotome and dermomyotome give rise to myoblasts which adopt more stringently defined fates and develop into distinct fiber types (Devoto et al., 2006; Gilbert, 2006).

Myogenesis is regulated at the level of the genome by an assembly of transcription factors collectively designated muscle regulatory factors (MRFs), along with the myogenic genes and cofactors (Relaix, 2006; Gilbert, 2006; Arnold and Winter, 1998). The genes and regulatory mechanisms which establish the myogenic lineage in the embryo remain largely the same throughout ontogeny, similarly fating cells to become myoblasts in larval (in the case of teleosts) growth and into adulthood (Marschallinger et al., 2009; Relaix, 2006). In addition to the genes encoding the MRFs, which include *Mrf4*, *Myf5*, *MyoD*, and *myogenin*, myogenesis is also critically dependent upon *Pax3* and *Pax7*, which appear to function upstream of the MRFs (Kablar et al., 1998; Devoto et al., 2006; Arnold and Winter, 1998; Marschallinger et al., 2009; Rescan et al., 1999; Relaix, 2006).

The process of myoblast formation, both during embryogenesis and in adult life, exists along a continuum as stem cells and muscle precursor cells undergo proliferation and differentiation. The MRFs are differentially expressed in accord with these cellular processes, each functioning in different capacities to regulate progression through the cell cycle and, in the case of mature myocytes, terminal differentiation (Chauvigné et al., 2005; Rescan et al., 1995; Rescan et al., 1999; Hinits et al., 2009). The individual MRFs may variously serve overlapping or complementary functions, in which several may operate in unison to mediate a particular phase along the myogenic pathway, or mediate processes at discrete points as the cell transitions out of the cell cycle to a differentiated state (Chauvigné et al., 2005; Arnold and Winter, 1998). In elucidating the developmental state of skeletal muscle the expression of *MyoD* and *myogenin* have frequently been exploited, as these genes are widely held to be fundamentally involved in

the early determination, and subsequent differentiation, respectively, of myoblasts (Steinbacher et al., 2007; Arnold and Winter, 1998).

As the myotome and dermomyotome emerge from the somite as morphologically and functionally delineated entities the expression of *Pax3* and *Pax7* which originally initiated the myogenic cascade is suppressed, replaced by an increased expression of select MRFs, notably, *Myf5*, *Mrf4*, and *MyoD*; the expression of these genes is followed soon after by that of *myogenin* (Relaix, 2006). The respective onset of the expression of *MyoD* and *myogenin*, both over the course of the commitment and differentiation of myoblasts and relative to one another, has resulted in the two genes being relegated to distinctly different roles during myogenesis (Hinits et al., 2009; Arnold and Winter, 1998; Bergstrom and Tapscott, 2001). While *MyoD* is regarded as functioning in the early stages of myocyte development by initiating myogenic determination, *myogenin* is considered to mediate differentiation of the mature myocytes; though these functions may exhibit some plasticity between vertebrate taxa (Delalande and Rescan, 1999; Hinits et al., 2009; Arnold and Winter, 1998; Bergstrom and Tapscott, 2001). However, both *MyoD* and *myogenin* are indispensable transcription factors throughout myogenesis, and both belong to the closely related group of basic helix-loop-helix (bHLH) proteins responsible for mediating skeletal muscle development (Bergstrom and Tapscott, 2001).

The precise temporal boundaries within which *MyoD* and *myogenin* function during embryonic myogenesis and muscle growth later in ontogeny currently remain somewhat obscure. Though they are responsible primarily for specification and differentiation respectively, there exists the potential for overlapping or shared functionality, which is made greater by the evolutionary differences between vertebrate

taxa (Hinits et al., 2009; Relaix, 2006; Delalande and Rescan, 1999; Bower and Johnston, 2010a). While the purview of the two transcription factors may fluctuate between species, and even throughout ontogeny, there exist profound structural differences between the two proteins that dictate their abilities to mediate the processes of specification and differentiation (Bergstrom and Tapscott, 2001). Both MyoD and myogenin retain similar bHLH domains which are responsible for DNA binding, however, variations in an amphipathic alpha-helix in the carboxy-termini of the two peptides endow highly distinctive transcriptional capacities (Bergstrom and Tapscott, 2001; Arnold and Winter, 1998). The alpha-helix contained within MyoD enables the transcription of specific myogenic genes in myoblasts, while in myogenin the alpha-helix performs as a less discriminating gene transcription domain (Bergstrom and Tapscott, 2001). This structural difference provides a mechanistic foundation for the role of MyoD in determination and that of myogenin in differentiation, and enables meticulous regulation of cellular fate by means of the disparate transcriptional activities executed by these myogenic proteins (Bergstrom and Tapscott, 2001).

The complexity of skeletal muscle development is made greater in teleosts by the duplication of the genome, and in the case of salmonids a tetraploidization, and the paralogs which evolved and experience differential regulation and selection (Macqueen and Johnston, 2006; Delalande and Rescan, 1999; Bower and Johnston, 2010a; Rescan et al., 1994; Rescan et al., 1995; Hinits et al., 2009; Rescan et al., 1999; Chauvigné et al., 2005). The evolution of numerous MRF paralogs allowed for specialization of function as individual genes were subjected to distinct selective pressures and genetic variation (Macqueen and Johnston, 2006; Bower and Johnston, 2010a; Delalande and Rescan,

1999). Expansion of the genome permitted paralogs to adopt unique purposes, and the presence of multiple gene copies facilitated the retention or loss of function with relative impunity as some paralogs may operate redundantly; the preservation of a mechanism by one enables an expansion in utility in another. In Atlantic salmon (*Salmo salar*) the various *MyoD* paralogs are differentially regulated as myoblasts undergo proliferation and differentiation; moreover, this differential regulation is additionally influenced by amino acid availability and the nutritional status of the fish (Bower and Johnston, 2010a). The paralogs have evolved to mediate different phases of myocyte development, becoming involved in the regulation of cell populations and fate, and responding to other physiologies such as metabolism as they are influenced by environmental variation (Bower and Johnston, 2010a). Similarly, *myogenin* and multiple *MyoD* paralogs experience differential expression not only as the somites develop, but within different regions of the somites themselves (Rescan et al., 1999; Delalande and Rescan, 1999). Further regulatory control is exerted by the promoter elements within *myogenin*, which are responsible for specific functionality in the skeletal muscle (Du et al., 2003). Successful transcriptional activation of myogenic genes by MyoD is additionally dependent upon Pbx homeodomain proteins, embellishing epigenetic regulatory control by ancillary factors involved in muscle development (Maves et al., 2007). Additionally, the various MRFs are differentially regulated temporally and spatially throughout skeletal muscle formation as the somites give rise to the myotome and dermomyotome, and cell lineages are increasingly restricted (Chauvigné et al., 2005; Bower and Johnston, 2010a; Hinitz et al., 2009; Rescan et al., 1999). Consistent with the roles of MyoD and myogenin in specification and differentiation, respectively, *myogenin* expression was

observed subsequent to that of *MyoD* paralogs in rainbow trout (*Oncorhynchus mykiss*) (Delalande and Rescan, 1999).

Among the defining morphological features of teleost skeletal musculature is the spatial delimitation of the fiber types, which is instructed by the differential expression of the MRF genes, as well as cell migration and cytokine signaling in the embryo and adult tissue (Henry and Amacher, 2004; Hirsinger et al., 2004; Devoto et al., 1996; Rescan et al., 1995; Hinitz et al., 2009; Rescan et al., 1999). The development of fast muscle fibers in zebrafish (*Danio rerio*) relies upon the prior formation of slow fibers and the *hedgehog* signaling which directs it (Henry and Amacher, 2004). The medio-lateral movement through the somite of muscle cells with a slow phenotype acts in a permissive fashion, enabling fast muscle fibers to differentiate in a trailing fashion (Henry and Amacher, 2004). The relative positions of cells within the somite similarly influence the fiber phenotype as cell migration in the myotome and dermomyotome leads to the development of fiber types from cells which have taken up residence in the various regions of these embryonic structures (Stellabotte et al., 2007). Indeed, aggregations of cells were discovered in zebrafish which were defined by gene expression characteristic of different fiber types even before the formation of somites (Devoto et al., 1996).

The wholesale migration of populations of cells, and reconfiguration of somites, are integral not merely for embryonic and early life stages, but for providing a foundation for growth potential in later life as well (Marschallinger et al., 2009; Steinbacher et al., 2007; Hollway et al., 2007; Stellabotte et al., 2007). Post-embryonic muscle growth in fish occurs through both hypertrophy and hyperplasia of the muscle, hyperplastic growth occurring in mosaic and stratified morphologies within the myotomes (Mommensen, 2001;

Johnston et al., 2010). Stratified hyperplasia takes place as the distal regions of the dermomyotome deposit new layers of cells in the trunk musculature as they move laterally from the notochord; while mosaic hyperplasia creates a heterogeneous landscape of muscle fibers as satellite cells scattered throughout the myotome differentiate into new myocytes, or are incorporated into existing myofibers as they grow in size (Johnston et al., 2010).

Hyperplastic growth in post-embryonic stages necessitates populations of satellite cells which are competent to continue proliferating, furnishing daughter cells for new myofibers as well as maintenance of their own populations (Johnston et al., 2003; Johnston et al., 2010). Dermomyotome-derived myogenic precursor cells distinguished by the expression of *Pax7* appear to function as precursor cells available for hyperplastic growth of fast muscle fibers later in ontogeny (Marschallinger et al., 2009); while hyperplasia of slow muscle fibers may occur by means of different mechanisms at various points in development (Barresi et al., 2001). In more derived vertebrates various mechanisms for securing populations of satellite cells for continued growth have been proposed (Gros et al., 2005; Schultz, 1996), while in zebrafish a dramatic reorganization of the somite has been demonstrated to give rise to a novel population of muscle progenitor cells (Hollway et al., 2007). For species exhibiting indeterminate growth, such as salmonids, the inherent value of populations of cells which are capable of continuous proliferation commensurate with the demands imposed by continuous hyperplasia is apparent. The addition of skeletal muscle mass through an increase in fiber numbers demands that satellite cells constitutively produce new cells while simultaneously preserving a population of stem cells which is not exhausted.

The physiology and regulation of myogenesis in salmonids is rendered more complex due to the genome tetraploidization that generated paralogs of many of the MRFs involved in muscle development. The evolution of these paralogs permitted more specialized and highly regulated mechanisms for muscle development to emerge. This in turn led to an expansion of the potential functions which might be fulfilled by these genes as it made possible the potential for them to take on novel functions or regulatory control. This would have been essential for a group such as the salmonids, which inhabit diverse environments and exhibit substantial growth potential. It also made possible the heterochronic manipulation of the various paralogs, which would have had implications for the radiation of genera and species, and modes of growth. The potential for the evolution of redundant and novel functions alike within myogenic genes was enhanced by increasing the genetic framework in which selection could operate, simultaneously providing a means by which to elaborate regulation of muscle development. Additionally, an indeterminate mode of growth makes possible a broader range of functions for the MRFs throughout ontogeny. Genes that serve in a particular capacity at a discrete point in development may execute similar or highly unique functions at a later point, a potential which represents distinctive opportunities in continuously growing species such as salmonids.

Growth hormone/IGF

Somatic growth, as with any prevailing physiological process, is profoundly complex and extensively regulated. It must be integrated with the multitude of other physiologies occurring simultaneously, and in such a way that it proceeds optimally while at the same time not detracting from other, sometimes competing, processes.

Additionally, growth is assimilated with an organism's environment, and the functioning of metabolic paradigms such as heterothermy and homothermy are differentially impacted by the extrinsic surroundings. Growth may be considered to occur from the point of the initial division of the fertilized egg, and as such, is imbued with tremendous complexity as growth physiology changes continuously throughout ontogeny. While in many organisms sexual maturity heralds the conclusion of somatic growth, other species grow indeterminately and continuously throughout their lifetime. Moreover, cell and tissue replacement occurs in all organisms at all points in ontogeny. The physiology of growth adopts a novel and utterly divergent significance in the context of deregulation and disease. Unrestrained growth is the hallmark of tumorigenesis and metastasis, wherein cells, often cancerous, may proliferate unchecked.

While growth may be considered to be directed by a staggering array of ligands and ancillary molecules, somatotropin (growth hormone, GH), insulin, and the insulin-like growth factors I and II (IGF-I and IGF-II) may be regarded as the fundamental molecular effectors of somatic growth, which encompasses myogenesis (Hadley and Levine, 2007; Florini et al., 1996; Wood et al., 2005; Duan, 1997). The somatotrophic axis is principally comprised of the hypothalamus, the hypophysis, and the liver, along with associated ligands including, but not limited to, growth hormone-releasing hormone (GHRH), somatostatin, somatotropin, IGF-I and -II, and insulin-like growth factor binding proteins (IGFBPs), as well as receptors, which have been found throughout vertebrate clades, including in teleosts (Florini et al., 1996; Moriyama et al., 2000; Björnsson, 1997). Historically, somatic growth was considered to be mediated exclusively by hepatically derived IGFs, the release of which was stimulated by growth

hormone originating in the hypophysis, though more recent evidence has inspired a reevaluation of this canonical view (LeRoith et al., 2001; Yakar et al., 1999; Sjögren et al., 1999).

The functioning and activities of the ligands within the somatotrophic axis are acutely dependent upon, and responsive to, not only the internal physiology but also the environment and a number of abiotic factors (Johnston et al., 2003; Johnston, 2006; Björnsson, 1997). Teleosts, as heterothermic vertebrates, are profoundly impacted by temperature, which influences growth throughout ontogeny (Gabillard et al., 2003; Johnston et al., 2011; Johnston, 2003). Growth and metabolic activities are swayed by ambient temperature, photoperiod, nutrient availability and feeding, as well as oxygen concentrations, all of which have been discovered to dramatically impact growth physiology and the expression profiles of growth related genes in teleosts (Johnston et al., 2003; Taranger et al., 2006; Chauvigné et al., 2003; Oakes et al., 2007; Johnston, 2006; Ren et al., 2010). Environmental cues such as photoperiod are received by the central nervous system and translated into the release of somatotropin, and have been demonstrated to elicit alterations in growth, and myogenesis specifically, and sexual maturity; thus illustrating the malleability of the phenotypic response and its susceptibility to extrinsic stimuli (Johnston et al., 2003; Taranger et al., 2006). Similarly, the availability of food resources and the nutritional status of an organism fluctuates continuously, which influences growth and metabolism on a temporal continuum, ranging from hourly to seasonal to annual cycles (Erbay et al., 2003; Oakes et al., 2007, Bower et al., 2008; Wood et al., 2005). In an experimental context the expression profiles of genes in the somatotrophic axis have been observed to differ intensely as fish were fasted

and subsequently fed to satiation (Bower et al., 2008; Chauvigné et al., 2003; Montserrat et al., 2006), and similar transcriptional control can be envisioned as feeding opportunities in wild fish populations are subject to prey availability.

Postnatal growth in vertebrates relies on the functionality of growth hormone and the suite of cellular processes it mediates; the central function of this endocrine factor is evidenced by its emergence early in vertebrate evolution (Kawauchi and Sower, 2006; Hadley and Levine, 2007). Growth hormone operates in concert with, as well as independently of, other effectors within the somatotrophic axis, participating in carbohydrate, lipid, and protein metabolism along with a host of other cellular workings (Wang et al., 2004; Florini et al., 1996; LeRoith et al., 2001; Lindén et al., 2000). The preponderance of information regarding the mechanisms of action and regulation of growth hormone have been derived from research carried out in mammalian species; while structural and/or functional homology cannot explicitly be inferred for fish, specifically teleosts, a number of the principal components and mechanisms are common to both (Kawauchi and Sower, 2006; Johnston et al., 2011; Björnsson et al., 2002) and the similarities and differences will only be better resolved as new research brings further insight. Growth hormone is synthesized in, and secreted from, the somatotrophs of the adenohypophysis, the stimulus and suppression of its release mediated in part by growth hormone-releasing hormone and somatostatin, respectively, from the hypothalamus (Klein and Sheridan, 2008; Hadley and Levine, 2007). Additionally, gastric and intestinal ghrelin have been found to stimulate growth hormone release in mammals and teleosts alike (Wang et al., 2002; Hadley and Levine, 2007). Growth hormone binding of the growth hormone receptor initiates intracellular signaling cascades responsible for the

regulation of downstream genes in the somatotrophic axis and other metabolic pathways (Herrington et al., 2000; LeRoith et al., 2001; Herrington and Carter-Su, 2001).

Sharing a degree of amino acid sequence homology with related receptors, the growth hormone receptor belongs to the cytokine/hematopoietin superfamily of receptors (Herrington and Carter-Su, 2001). The growth hormone receptor contains a single extracellular domain as well as sole transmembrane and cytoplasmic domains, and while homology of the receptor within vertebrate clades is relatively low, the extracellular domain remains an exception, exhibiting substantial homology (Björnsson et al., 2002; Argetsinger and Carter-Su, 1996). The extracellular domains of the mammalian receptors thus far characterized contain seven cysteine residues, six of which are associated through disulfide bonds; salmonid receptors, however, possess only four of these cysteines (Björnsson et al., 2002; Argetsinger and Carter-Su, 1996). A single domain of the growth hormone ligand initially binds to the extracellular domain of a single growth hormone receptor, followed sequentially by the recruitment and binding of a second receptor to a domain on the ligand distinct from the one which bound the initial receptor (Cunningham et al., 1991).

While it remains uncertain if this binding induces a conformational change in the receptor-ligand complex, growth hormone binding does solicit association of intracellular Janus kinase 2 (JAK2) peptides with the intracellular domains of the dimerized receptors at a conserved region dominated by prolines in close proximity to the membrane, termed Box 1 (Herrington and Carter-Su, 2001). The bound JAK2 proteins reciprocally phosphorylate one another on discrete activating tyrosine residues, and subsequently phosphorylate themselves as well as the growth hormone receptor, thereby creating an

active binding site for intracellular signaling molecules (Herrington and Carter-Su, 2001). Certain of the signal transducer and activator of transcription (STAT) proteins are among those known to bind the activated growth hormone receptor complex, and do so through their SH2 (Src (sarcoma) Homology 2) domains (Argetsinger and Carter-Su, 1996). The STATs activated by the growth hormone receptor cascade subsequently complex with one another and translocate to the nucleus, regulating transcription of downstream genes (Ihle, 1996). In addition to transducing signals through STAT proteins, growth hormone binding of its receptor initiates mitogen activated protein kinases (MAPKs) such as extracellular signal regulated kinases (ERK)-1 and -2 (Argetsinger and Carter-Su, 1996). The insulin receptor substrates (IRS)-1 and -2 are also mediated by growth hormone receptor activation, which, through SH2 binding of phosphoinositide 3-kinase (PI3K), lead to metabolic regulation of lipogenesis and most likely glucose transport by PI3K (Argetsinger and Carter-Su, 1996). While the array of tyrosine residues in the intracellular domain of the salmonid receptor has been found to differ from that of other vertebrates (Björnsson et al., 2002) the JAK-STAT signaling cascade initiated by growth hormone appears present in teleosts (Lee et al., 2001). Entrenched in a multitude of signaling pathways and anabolic mechanisms, growth hormone is an integral and dynamic component of growth physiology. However, along with these many functions is the endocrine regulation of, and interaction with, the insulin-like growth factors.

Equally, and in some contexts perhaps more, imperative to growth in vertebrates are the insulin-like growth factors-I and -II (IGF-I and IGF-II), their associated receptors, binding proteins, and coordinate molecules such as the acid-labile subunit (ALS) (Wood et al., 2005; Björnsson et al., 2002; Sjögren et al., 1999). The vertebrate IGFs are fairly

well conserved structurally and the mammalian ligands resemble immature insulin, containing four unique domains, B, C, A, and D (Duan et al., 2010). The IGFs are initially synthesized in a prohormone conformation, containing a hydrophobic signaling domain that mediates secretion, which is subsequently cleaved but leaves the hormones in an immature form as an E domain remains (Wood et al., 2005). The mature forms of the IGFs emerge as this E domain is proteolytically cleaved (Moriyama et al., 2000).

The IGFs are bound by two distinct receptor types, IGF-I receptors (IGF-1R) and IGF-II receptors (IGF-2R), which execute both confluent and unique signaling mechanisms in fish and mammals alike (Duan et al., 2010; Wood et al., 2005). The IGF-1R is cleaved following initial translation as a single peptide into distinct α and β subunits of approximately similar sizes, which are then bound to one another through disulfide bonds, forming an $\alpha\beta$ hemireceptor (Wood et al., 2005). This partial receptor subsequently associates with another identical hemireceptor, giving rise to the functional $\alpha_2\beta_2$ holoreceptor (Butler et al., 1998). Ligands, primarily IGF-I, are bound by the extracellular α domain, while the cytosolic β domain carries out phosphorylation as the tyrosine kinase domain, and defining the IGF-1R as a member of the tyrosine kinase family of transmembrane receptors (Florini et al., 1996; Wood et al., 2005). A conformational change in the IGF-1R brought about by ligand binding is translated into activation of the β domain through autophosphorylation of discrete tyrosine residues (Butler et al., 1998; Florini et al., 1996).

Activation of the β domain initiates several distinct signaling cascades as multiple intracellular effectors are drawn in to the active site of the cytoplasmic IGF-1R domain (Wood et al., 2005). The insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) are the

preeminent signaling factors recruited to the β domain, and the IGF-1R is somewhat unique among the tyrosine kinase receptors in that IRS-1 and IRS-2 are utilized as intermediaries between the activated IGF-1R and several secondary intracellular substrates involved in different pathways (Butler et al., 1998). Deficiencies in IRS function prove catastrophic to the coordination and perpetuation of downstream signaling in muscle tissue (Long et al., 2011). Intracellular messenger proteins containing SH2 domains are among those enlisted by the IGF-1R to communicate IGF-I binding to the Ras/MAPK pathway; IGF-I binding additionally initiates PI3K and Akt/mammalian target of rapamycin (Akt/mTor) pathways through IGF-1R mechanisms (Duan et al., 2010; Wood et al., 2005).

These cascades, while commonly initiated by IRSs, and ultimately IGF-I, are variously involved in processes such as metabolism and nutrient utilization, and transcriptional activation related to growth (Florini et al., 1996; Butler et al., 1998). IGF-I signaling promotes somatogenesis by regulating contrary metabolic processes simultaneously, stimulating anabolic pathways while antagonizing the expression of genes involved in protein degradation (Cleveland and Weber, 2009; Seiliez et al., 2010; Stitt et al., 2004). Transcription of the muscle RING-finger proteins (MuRFs), which are involved in ubiquitination and degradation of muscle protein, is carried out by FOXO (Forkhead box) transcription factors (Witt et al., 2005; Stitt et al., 2004). IGF-I antagonizes FOXO nuclear translocation and transcriptional activation of MuRFs, thereby preventing breakdown of muscle tissue by ubiquitin ligases (Stitt et al., 2004; Seiliez et al., 2010). The PI3K and Akt pathways which are recruited for anabolic metabolism are similarly enlisted in down-regulating these ubiquitin ligases through

interference with the actions of FOXO effectors, operating in teleosts and mammals alike (Cleveland and Weber, 2009; Stitt et al., 2004; Seiliez et al., 2010).

In contrast to the varied responses mediated by the IGF-1R the type II IGF receptor has, at present, not been found to serve in any signaling pathway; indeed, the IGF-2R appears to be without any catalytic motif or capacities, though is requisite for survival (Duan et al., 2010; Wood et al., 2005). While the affinity of the mammalian IGF-2R for IGF-II tremendously exceeds that for IGF-I, binding of IGF-II initiates endocytosis and degradation of the receptor-ligand complex rather than signal transduction (Duan et al., 2010). The IGF-2R has, however, been established in mammals as being identical to a mannose 6-phosphate (M6P) receptor (Duan et al., 2010). While the IGF ligands and receptors have been perhaps most extensively studied in mammals their structures and/or physiological functions have remained reasonably conserved with those in teleosts (Pozios et al., 2001; Duan et al., 1997; Wood et al., 2005; Moriyama et al., 2000).

The IGF-1R has been described in a number of teleost species, including rainbow trout (Greene and Chen, 1999), and as the IGF-1R signaling pathways are elucidated in teleost species it is becoming clear that they are homologous with those of mammals (Pozios et al., 2001; Wood et al., 2005); notably, a β domain tyrosine kinase has been found to be present in rainbow trout (Greene and Chen, 1999). Unlike mammals, the zebrafish IGF-1R bound both IGF-I and IGF-II in a comparable manner (Pozios et al., 2001). An IGF-2R with binding specificity for the IGF-II ligand has been found in brown trout (*Salmo trutta*) (Mendez et al., 2001), however, this work was performed with embryos rather than fish at later ontogenetic stages. IGF-II signaling fulfills functions

unrelated to metabolism in zebrafish during embryonic development (White et al., 2009), suggesting differential roles at various points during the life history of the species. While the structures and pathways of the IGF system have exhibited conservation across vertebrate taxa the numerous commonalities do not explicitly imply universal homology in every aspect of expression, function, or mechanism of IGF-mediated growth (Wood et al., 2005).

The somatotropic axis and its attendant components are accountable to stringent regulation at myriad points both as a function of the physiology of the organism, and along a temporal continuum. Growth hormone, IGFs, receptors, and stimulatory factors, both positive and negative, operate within the somatotropic axis at multiple levels of organization throughout ontogeny. Growth hormone and the IGFs are strictly moderated by feedback mechanisms as are all endocrine effectors. Excitatory and inhibitory stimuli originating in the external environment or internal physiology regulate the production of factors which alternately promote or antagonize the release of growth factors. Somatostatin ceases growth hormone release (Klein and Sheridan, 2008), in addition to exerting control over hepatic IGF-I expression (Very et al., 2008), while growth hormone-releasing hormone stimulates ligand production and excretion. The tissues responsible for these effectors incorporate signals pertaining to the nutritional status, environmental conditions, and developmental stage of the organism and respond accordingly. Growth hormone in turn functions to mediate its anabolic activities both directly and indirectly (Wang et al., 2004), thereby simultaneously serving as a regulator of IGFs as well as itself as feedback to the adenohypophysis and hypothalamus promotes or halts further synthesis. The mechanisms relating information about the concentration

of hormones present in the organism may all be considered to operate through cyclical feedback, though they may occur on spatial scales varying in scope. Paracrine and autocrine regulation of growth factors contributes to the command of growth factors, and in some contexts may prove to supersede endocrine actions (Yakar et al., 1999).

The distribution and regulation of growth hormone and IGF receptors has proven to be another mechanism operating in concert with the endocrine regulation of these ligands to dictate the response of cells and tissue to various growth stimuli; the liver contains abundant growth hormone receptors though many other tissues contain the receptor as well (Flores-Morales et al., 2006; Wood et al., 2005). Muscle-specific cofactors are requisite for the maintenance and turnover of IGF receptors (Demonbreun et al., 2010) while intracellular protein interactions provide a mechanistic basis for moderating growth hormone signaling pathways (Flores-Morales et al., 2006). Growth hormone signaling, like that of myostatin and innumerable other ligands, is governed by receptor expression, turnover, and degradation (Flores-Morales et al., 2006). Signaling through the growth hormone receptor is dependent in large part on the suppressors of cytokine signaling (SOCS), a group of related proteins some of which act specifically on the JAK-STAT pathway impelled by growth hormone binding, promoting ubiquitination and degradation of the JAK and STAT proteins involved in signal transduction (Flores-Morales et al., 2006).

Paramount to the endocrine and paracrine actions of IGFs are the insulin-like growth factor binding proteins (IGFBPs), with at least six distinct peptides currently characterized in mammals (Duan, 1997). These proteins are characterized by the presence of shared motifs and outstanding affinities for IGF-I and IGF-II, though remain

unique with respect to certain of their structural and functional attributes (Duan and Xu, 2005; Clemmons, 2001). The IGFBPs serve in a variety of capacities, including safeguarding circulating IGFs from proteolytic cleavage or degradation and thereby extending their viability (Duan et al., 2010; Wood et al., 2005). Additionally, they appear to participate in making the IGFs available to tissues in a spatially discrete fashion by sequestering them in localized regions through binding them within complexes from which they can be released, as well as negotiating their movement from the circulation to the receptors on the cell surface (Duan, 2002). While the multiple IGFBPs fulfill diverse physiological necessities a complex incorporating IGFBP-3 and a glycoprotein, the acid-labile subunit (ALS) figure prominently in the regulation of mammalian IGFs and their somatic transport (Wood et al., 2005). These IGFBP interactions evidence mechanisms resembling paracrine and autocrine control of ligand availability and may represent a means of satisfying the varying requirements of different tissues for these growth factors (Duan, 2002).

As a remarkably dynamic and highly involved process somatic growth necessarily represents the culmination of the interaction of a host of molecules. Figuring prominently in this physiology, the IGFs and growth hormone have consistently been the focal points on which elucidating growth processes has centered. However, the precise nature of their interaction, and the manner in which each functions dependently or independently to mediate growth, remains the subject of some debate. Initially, the model proposed installed hypophyseal growth hormone as the consummate mediator of growth and anabolic metabolism as it stimulated the hepatic synthesis and release of the IGFs, which subsequently functioned as the ultimate growth promoting factors

(Schlechter et al., 1986; Butler and LeRoith, 2001; LeRoith et al., 2001). Growth hormone was interpreted to operate through the hepatically-derived IGFs, rather than directly, to stimulate growth and early investigations demonstrating direct growth hormone actions overlooked the tissue specific capacity for initiating IGF actions (Isaksson et al., 1982; LeRoith et al., 2001). As these hormones became better understood and their discrete regulation and expression were better resolved the paradigm shifted, recasting growth hormone and the IGFs in both independent and dependent roles (LeRoith et al., 2001; Wang et al., 2004; Lupu et al., 2001). IGF expression was found to be all but ubiquitous, no longer held as exclusively the purview of hepatocytes, suggesting that tissue-specific autocrine and paracrine IGF actions had a previously unrealized significance (Björnsson et al., 2002; Yakar et al., 1999; Sjögren et al., 1999). While IGFs derived from the liver remain indispensable to a suite of physiological processes it was found that mammalian chondrogenesis was equally, if not more so, dependent upon locally derived IGFs (Ohlsson et al., 2009). The tissue specific expression of IGFs has exhibited the ability to rescue a normal growth phenotype in the absence of hepatically-derived IGFs, and indicated that paracrine and autocrine IGF actions, whether induced by growth hormone or independent of it, are fundamental to somatogenesis (Yakar et al., 1999; Sjögren et al., 1999; Tanaka et al., 1996).

In addition to the novel mechanisms by which the IGFs functioned in somatic tissues, independent actions for growth hormone which did not rely on the IGFs were also realized (Wang et al., 2004; Lupu et al., 2001). Definitive confirmation of the direct action of growth hormone on mammalian chondrogenesis could be seen in the growth deficiencies exhibited by double mutant *Ghr/Igf-I*-null mice, which were more severe

than those found in mice containing only a single mutation (Lupu et al., 2001). Similarly, *IGF-I*-null mice were found to have compromised hypertrophy of chondrocytes, while *Ghr*-null mice exhibited deregulation of cell proliferation and cell growth (Wang et al., 2004). These data collectively demonstrate autonomous roles for growth hormone in mediating growth, as well as pointing to the substantial redundancy and overlap which exists in the functioning of the IGFs and growth hormone (Lupu et al., 2001; Wang et al., 2004).

The improved understanding of these cardinal growth factors has revealed some of the mechanisms which underpin their stimulation of growth. Growth hormone and the IGFs were originally regarded as independently responsible for the disparate cellular process of proliferation and differentiation (LeRoith et al., 2001). Subsequently, this clear delineation for each in the regulation of cellular events has become blurred as it has been revealed that growth hormone and the IGFs participate in both proliferation and differentiation in varying capacities depending upon the context in which they are operating (Ren et al., 2010; LeRoith et al., 2001; Rosenthal and Cheng, 1995; Engert et al., 1996). The failure of both hypertrophic and hyperplastic growth in *Ghr*-null mice supports a role for growth hormone in both cell proliferation and differentiation (Wang et al., 2004). Additionally, IGFs are capable of alternately promoting differentiation or proliferation in myoblasts depending upon the oxygen concentration available to the cells and the signaling pathways enlisted (Ren et al., 2010; Coolican et al., 1997). The disparity in cell fates under normoxic and hypoxic conditions is mediated by the differential control of the numerous signaling pathways initiated by IGF binding; hypoxia leading to proliferation through activation of specific pathways while normoxia slates

cells for differentiation by means of alternative cascades (Ren et al., 2010; Duan et al., 2010). In this way identical ligands are able to execute two otherwise conflicting cellular processes (Ren et al., 2010). Growth hormone and the IGFs may now be considered to function both independently and in concert to regulate somatogenesis; the vastly expanded role for local IGF actions reveals that there is likely tremendous opportunity for spatially distinct regulation, facilitating more refined control of growth (Ohlsson et al., 2009; LeRoith et al., 2001).

The somatotrophic axis of teleosts differs minimally from that of other vertebrates with respect to major components such as the organs and endocrine factors involved, and some of the signaling pathways utilized (Wood et al., 2005). Growth physiology in teleosts has been intensively studied not only in an effort to understand the diversity and homology of these processes across vertebrate species, but because it represents a means of enhancing species valuable to the commercial industry (De-Santis and Jerry, 2007; Garber et al., 1995). With the advent of commercial aquaculture artificial selection has enhanced some of the components within the somatotrophic axis known to be essential to growth regulation (Tymchuk et al., 2009; Neregård et al., 2008).

Growth hormone has been utilized to enhance somatic growth in teleosts, most often salmonids and other commercially valuable species, through both the generation of transgenic organisms and the introduction of exogenous hormone (Garber et al., 1995; Levesque et al., 2008; Devlin et al., 2009; Devlin et al., 2004). The genetic and extrinsic manipulations of the somatotrophic axis have both resulted in similar alterations in gene expression, increasing anabolic pathways such as those involving IGF-I and myogenic factors, demonstrating at least minimal conservation of some of the fundamental

components of the vertebrate growth program (Biga et al., 2005; Levesque et al., 2008; Devlin et al., 2009; Biga et al., 2004a). As heterothermic vertebrates the metabolic demands of teleosts differ from those of mammals and other homeotherms, which potentially influences the somatotrophic axis in fish in unique ways. The expression profiles of genes within the somatotrophic axis in fish have been directly correlated to the nutritional status of the animal (Chauvigné et al., 2003; Amaral and Johnston, 2011; Oakes et al., 2007; Montserrat et al., 2006; Bower et al., 2008; Bower and Johnston, 2010b; Campos et al., 2009).

Nutrient deprivation and surfeit result in the differential regulation of the IGFs and their receptors (Erbay et al., 2003; Montserrat et al., 2006; Chauvigné et al., 2003), as well as those in the catabolic and degradation pathways (Amaral and Johnston, 2011). The two IGFs responded to nutrient availability with dramatically different expression, IGF-I typically exhibiting more dramatic variations in expression than IGF-II (Chauvigné et al., 2003; Montserrat et al., 2006). These findings suggest disparate functions for these growth factors in the response to nutrient flux and the regulation of anabolic and catabolic pathways; amino acid availability directly influencing the IGF-I response as muscle mass was augmented in Atlantic salmon (*Salmo salar*) (Bower and Johnston, 2010b). Moreover, the enhancement in growth brought about by growth hormone transgenesis or the administration of an exogenous peptide was the direct result of improvements in the utilization of nutrients and a metabolic shift towards muscle accretion and protein synthesis rather than lipid deposition (Garber et al., 1995; Oakes et al., 2007). This indicates that alterations to the somatotrophic axis yield a mechanism by which resources may be used more efficiently rather than simply requiring greater

volumes of them. This may be the product of refinements to anabolic pathways in the context of modified expression of somatogenic genes, or the result of some as yet undiscovered cellular mechanism that may improve growth physiology within discrete contexts.

Interestingly, some of the ligands foremost involved in myogenesis and growth physiology, myostatin and those of the growth hormone-IGF (GH-IGF) axis, have demonstrated manifest interactions and complementary gene expression (Liu et al., 2003; Roberts et al., 2004; Oldham et al., 2009; Biga et al., 2004b). Myostatin, a negative regulator of skeletal muscle in mammals and the GH-IGF axis serve diametrically opposing metabolic functions, perhaps accounting for the nature of the interaction between the two, which is characterized by a mitigating effect of growth hormone on *myostatin* expression and concentrations of the mature myostatin protein (Liu et al., 2003; Biga et al., 2004b; Oldham et al., 2009). Growth hormone administration in humans produced a significant decrease in *myostatin* expression, while *in vitro* exposure of myocytes to the peptide produced similarly significant reductions in expression (Liu et al., 2003). Moreover, as *growth hormone receptor* expression was experimentally inhibited (Liu et al., 2003), or declined with age (Marcell et al., 2001) a corresponding increase in *myostatin* expression was observed. In mammals, the pathways recruited by growth hormone to antagonize production of the mature myostatin protein differed from those involved in regulating expression of the gene, suggesting differential transcriptional and post-translational control (Oldham et al., 2009). Similar, although inverse, relationships have been found at other points along the GH-IGF axis as TGF- β 1 has been

found to interfere with the expression of *IGF-II* and synthesis of the protein, as well as IGF-I signaling, within muscle (Gardner et al., 2011).

Interactions between myostatin and the GH-IGF axis identical to those in mammals have also been described in teleosts (Roberts et al., 2004; Biga et al., 2004b). Increased *growth hormone* expression in transgenic coho salmon (*Oncorhynchus kisutch*) yielded reductions both in *myostatin* expression and the mature protein (Roberts et al., 2004). Supporting a direct interaction between the myostatin gene and growth hormone peptide is the presence of a sequence in the regulatory domain of both myostatin genes in brook trout (*Salvelinus fontinalis*) that is responsive to growth hormone (Roberts and Goetz, 2003). Moreover, the expression of the two myostatin genes in both oxidative and glycolytic muscle of salmonids was differentially regulated in the case of increased growth hormone levels, whether achieved through endogenous or exogenous means (Biga et al., 2004b; Roberts et al., 2004). These data indicate not only that the two genes function in disparate capacities in the regulation of myogenesis, but that their response to the GH-IGF axis is similarly unique (Roberts et al., 2004; Biga et al., 2004b). While the antagonism of myostatin protein and mRNA by growth hormone suggests that myostatin functions to inhibit myogenesis in teleosts as it does in mammals, and hyperplasia accompanies reduced *myostatin* expression coincident with growth hormone increases (Roberts et al., 2004; Biga et al., 2004b), there remains the possibility that the impact of the GH-IGF axis on myostatin is ancillary to other anabolic effects of growth hormone augmentation which remain dominant. The potential for interactions between these molecules at the transcriptional or translational levels remains intriguing as they may further understanding of the evolution of growth physiology in vertebrates.

Aquaculture

With a burgeoning global population continuing to increase with each passing year, ever more strain is being placed on finite and dwindling resources. Increasingly, the aquaculture industry and its attendant technologies are being looked to for viable solutions to the perpetually growing food demands imposed by expanding human consumption. Changing demographics and upward economic mobility have already substantially heightened demand for a variety of aquaculture products in many parts of the world (Gjedrem et al., 2012). The relative nature of many of these products as luxury items in many societies contrasts sharply with their role in poverty-stricken nations where they are relied upon in a subsistence context (Ewoukem et al., 2012; Gjedrem et al., 2012). Tragically, but perhaps not surprisingly, those in greatest need of stable and successful aquaculture infrastructure are the same who are most severely disadvantaged by disruptions to whatever framework may be in place in the event of natural disasters or similar occurrences (Rethinking Poverty, 2010). The escalating necessity and desire for aquaculture products worldwide is certain only to fuel expansion and elaboration of an industry already developing at an accelerated pace.

Commercial scale aquaculture embodies one of the more contemporary methods of food production for human consumption (Subasinghe et al., 2009), and as a fledgling industry is in the midst of refining and honing the technologies and practices which will almost certainly secure its place as the preeminent means of obtaining fish protein in the coming decades. Presently, cultured fish comprises more than one quarter of the fish brought to market (Naylor et al., 2000), a figure which is certain to swell alongside growing populations. However, expansion of the industry has been plagued by a number

of obstacles which must be resolved if the viability and profitability of aquaculture are to be cultivated.

Foremost among these challenges is the procurement of adequate supplies of feed, in particular for cultured species that rely primarily on fish protein (Naylor et al., 2000; Pelletier et al., 2009). While the greatest volumes of fish oil and fish meal are consumed in the production of poultry and pork protein, the aquaculture industry demands substantial amounts of the same for use in bringing finfish to market (Naylor et al., 2000; Péron et al., 2010; Pelletier et al. 2009). Supplying adequate feed represents one of, if not the, single greatest expenses in the operation of aquaculture facilities (Naylor et al., 2000). The fact that herbivorous and omnivorous species rely more heavily on plant-derived proteins than those from fish sources (Naylor et al., 2000) may, in coming years, lead to a reconsideration of aquaculture practices wherein such species are preferentially grown in favor of piscivorous fishes.

The tremendous cost and energies associated with securing fish meal and fish oil, as well as the dependence on fisheries targeting lower trophic level species, has encouraged considerable investigation into alternative sources of raw materials for feed production (Pelletier and Tyedmers, 2007; Pelletier et al., 2009; Naylor et al., 2000). While the metabolic physiology of teleosts enables them to conserve greater amounts of the protein taken in when compared to livestock species (Pelletier et al., 2009; Gjedrem et al., 2012) the sheer magnitude of aquaculture production, along with dwindling stocks of wild fisheries, preclude anything less than the most aggressive pursuit of alternative feed sources (Pelletier et al., 2009; Naylor et al., 2000; Péron et al., 2010).

A variety of alternatives to protein derived from fish sources have been investigated, including the remnants and discards from the processing of livestock such as bone and blood meal (Naylor et al., 2000). Plant protein sources, principally soy, corn, and canola oils, have come to the fore as prospective substitutions for fish meal and fish oil (Enterria et al., 2011; Collins et al., 2012). As sustainable practices are being realized as a mainstay for the aquaculture industry the origination of feed sources, traditional and alternative alike, and the environmental impacts of the individual constituents are being more severely scrutinized. Associated with each of the components of all types of feed are both the financial and environmental costs of procurement, processing, refinement, and transportation (Péron et al., 2010; Pelletier and Tyedmers, 2007; Pelletier et al. 2009; Subasinghe et al., 2009). Indeed, the environmental detriments wrought by current mechanisms of feed production are so cumbersome and disproportionately great as to obliterate any gains made by the use of organically derived materials (Pelletier and Tyedmers, 2007).

At the intersection of increased demand and the necessity to fulfill it in sustainable fashion lies the opportunity to seize upon classical, as well as more contemporary, techniques to advance both of these ends with singular efficacy. The installation and development of selective breeding programs has been championed as a means of enhancing the growth rates of species central to aquaculture (Gjedrem et al., 2012). Capitalizing on traits inherited in Mendelian fashion, aquaculturists have the means to improve broodstock and overall yield as the most desirable alleles and characteristics are identified and selected for (Gjedrem et al., 2012). Similarly, transgenic means hold vast potential to expedite this process and develop organisms

which are not only able to reach market size more rapidly, but do so with greater efficiency. A variety of genomic techniques have long been employed in aquaculture, as well as other contexts (Horváth and Obrán, 1995), while more recently transgenesis has demonstrably improved growth and metabolic physiology in salmonids (Oakes et al., 2007).

Presently, the AquaAdvantage[®] salmon, produced by AquaBounty Technologies[®], is vying to become the first transgenic animal product approved in the United States for human consumption. This fish, which experiences enhanced growth due to a constitutively expressed Chinook salmon (*Oncorhynchus tshawytscha*) growth hormone gene which is under the control of an ocean pout (*Zoarces americanus*) promoter is able to be harvested in substantially less time than other salmon (www.aquabounty.com). Despite the need for such innovative technologies, approval from the United States Food and Drug Administration has been withheld, and detractors of the transgenic salmon have been many and vociferous (Homer, 2011; Bennett, 2011; Bennett, 2011b).

While aquaculture has come to be widely regarded as a means of bolstering economic and social prosperity in developing and industrialized nations alike, the answer to the difficulties associated with it is not a monolithic entity. As with most technological advancements there are benefits and shortcomings unique to aquaculture, and every effort must be made to ensure that the industry to moves forward in a sustainable and responsible manner. Though researchers assert that aquaculture represents a means of complementing captures from wild fisheries, they caution that it will almost certainly not redeem these fisheries from their currently overexploited state (Naylor et al., 2000). Ultimately, however, aquaculture retains tremendous potential for expansion and the

practice of environmentally sound management. Moreover, it does much to advance the cause of ready access to a valuable food source, which remains an indispensable commodity now, and in the future.

Rainbow trout as a model organism

Rainbow trout have, and continue to be, of inestimable value as a model organism; their use as such rivaled only by that of zebrafish (*Danio rerio*). The sheer presence of the species within investigative research is made all the more impressive by the breadth of the research fields in which rainbow trout have been utilized. Recent decades have seen the use of these fish as the model organism of choice in a staggering number of research endeavors, owing to a variety of features of the organism itself (Thorgaard et al., 2002).

Surpassing many species, including a number of which that are closely related, rainbow trout have distinguished themselves as a highly sought after species for research, sport, and aquaculture purposes (Thorgaard et al., 2002). The fish remains among one of the most widely cultivated species in aquaculture, and many of the characteristics that have made it desirable to the aquaculture industry are the very same that have made it equally attractive and profoundly ubiquitous as a model for research (Thorgaard et al., 2002). The species' importance to both aquaculture and researchers has in fact fostered a self-sustaining reciprocity. The insight gained in the laboratory is instituted within the industry to improve the culture of the fish, and the practices which are commonplace in the industry, along with increasing demand for the fish, in turn serve as an impetus for continued research into the organism.

While zebrafish, fugu (*Takifugu rubripes*), and medaka (*Oryzias latipes*) are all extensively used in countless areas of research, rainbow trout have rightfully commanded the attentions and efforts of investigators. A suite of life history, anatomical, and physiological features of rainbow trout, along with the extensive historical cultivation of the species, have elevated these fish to the highest echelons of model organisms (Thorgaard et al., 2002). Relative to species such as medaka and zebrafish, rainbow trout maintain a greater body size throughout their ontogeny, which permits surgical and physical manipulation which is impossible or excruciatingly difficult in smaller fish (Thorgaard et al., 2002). Endocrinological investigations may be more easily realized in a species such as rainbow trout as their body size is more amenable to canulation and greater volumes of plasma may be obtained. Similarly, investigation into individual tissues is a more practical reality in an organism in which ample volumes of such tissues are available and ready access to them may be achieved (Bunton, 1996). Ablation and replacement investigations may be accomplished not only by molecular means, but by physical manipulation in a rainbow trout, a feat not so readily achieved in smaller species.

The intensive energies focused on cultivating and rearing rainbow trout for aquaculture and sport purposes have translated to a tremendous body of knowledge regarding the maintenance and care of these fish (Thorgaard et al., 2002). Such an extensive understanding has further encouraged their use as a model organism and made laboratory rearing of the fish a commonplace and highly manageable practice (Thorgaard et al., 2002). The exceptional fecundity of these fish, along with the more minimal pecuniary demands and facility requirements necessary for maintaining large numbers of

these fish has only served to make them more desirable as a model system (Powers et al., 1989). Additionally, the intricacies of every stage of the life history of these fish is well known (Powers, 1989; Thorgaard et al., 2002), facilitating not only successful perpetuation of the fish in the laboratory, but exploitation of all phases of the development and ontogeny of the species. Admittedly, the comparatively longer reproduction cycles and time required to reach maturity may limit the rate with which some investigations proceed; however, this may be counterbalanced by the advantages afforded by the larger size of the gametes and embryos (Bailey et al., 1996; Thorgaard et al., 2002). The production of transgenic rainbow trout has, without question, benefited from the ease of microinjection of DNA constructs afforded by the large size of the gametes and visual identification of the micropyle.

Considered in a macroscopic light rainbow trout prove advantageous as they are, ultimately, a vertebrate model, albeit a more basal one. Prominent among the studies performed in rainbow trout that have implications for human health are those concerned with the immune system (Thorgaard et al., 2002). Many of the benefits afforded by rainbow trout, and indeed all vertebrate models, may be found in the ease with which they may be used as a proxy not only for vertebrate physiologies and systems, but for human disease and pharmaceutical intervention. Rainbow trout and other fish models have become widely used in toxicity and carcinogenicity studies for a host of reasons, among them the inherently negligible incidence of spontaneous tumorigenesis in these fish (Bailey et al., 1996; Law, 2003; Kissling et al., 2006; Scarpelli et al., 1963; Nunez et al., 1991). Fish models, and rainbow trout in particular, have served as a superior research platform for carcinogenicity studies exactly because they can readily be

introduced to potentially carcinogenic agents by means of exposure in water, and represent the vertebrate condition (Bailey et al., 1996, Powers, 1989). Rainbow trout offer the opportunity to examine the impacts of carcinogenic agents throughout their ontogeny as exposure may be introduced at any point from embryogenesis forward (Bailey et al., 1996). Increased resolution of oncogenetic events is provided in models such as trout as they have demonstrated a heightened sensitivity to carcinogenic and toxic compounds (Bunton, 1996).

The minimal rates of endemic tumorigenesis in fish such as trout served to draw immediate scrutiny when increased levels of hepatic neoplasms were found among hatchery raised fish (Scarpelli et al., 1963; Nunez et al., 1991). Investigation into this epidemic found aflatoxin B₁ to be the causative agent, but perhaps more importantly, demonstrated the usefulness of rainbow trout in carcinogenicity studies (Nunez et al., 1991). Further benefit from the use of rainbow trout in such investigations is derived from the ease with which large numbers of fish may be obtained and subjected to treatment with compounds under investigation (Bailey et al., 1996). Investigations utilizing such substantial numbers of study participants dramatically increases the statistical validity of their findings (Bailey et al., 1996), and the use of nonmammalian subjects has appeased a public which finds the use of mammalian species as research subjects aesthetically repugnant (Law, 2003).

While rainbow trout and other fish models have historically served as viable models for cancer research, and continue to do so presently, certain cautions are urged. Kissling et al. (2006) note that extrapolating the findings of carcinogenicity studies in fish to mammals should be done with important caveats, including the differential sensitivity

of teleosts and mammals to the compounds under investigation. These researchers cite the importance of utilizing fish models in such studies in conjunction with the use of mammalian models, rather than in place of them, noting that the organismal milieu may prove sufficiently disparate to preclude unqualified comparison. Similarly, the lack of tissue homology between fish and mammals means that the findings of cancer research performed with rainbow trout must be interpreted with full understanding of the significant phylogenetic distance between the two, and the attendant implications (Bailey et al., 1996; Bunton, 1996).

Among the suite of benefits afforded by the use of the rainbow trout as a model organism is the extensive degree to which it may be used to answer inquiries into vertebrate biology and evolution with great efficacy. The phylogenetic position occupied by these fish represents both hindrance and help with respect to vertebrate studies. While significant insights may be gleaned from work with these fish regarding vertebrate physiology and biochemistry they remain a more basal vertebrate. Their ability to approximate the mammalian conditions is somewhat constrained and they must be considered a highly useful model, but one which cannot stand independent of others. The evolutionary rift between these basal teleosts and mammals, and humans in particular, conversely offers a variety of advantages as well.

The functioning of systems and physiologies in these fish provides a frame of reference for the evolutionary development of molecules and pathways common to vertebrate lineages. The ability to render these comparisons enables understanding of how environment influenced evolutionary pathways, and how organ systems and subcellular macromolecules have alternately been conserved or repurposed for events

such as the invasion of terrestrial environments and homeothermy. Rainbow trout also provide a means for elucidating evolutionary differences even within teleost lineages as findings derived from their study may be compared to those of more derived species such as fugu. The degree of conservation in the structure and function of essential signaling molecules such as hormones may be better characterized as they are compared between trout and murine model organisms and, ultimately, humans. The evolution of indispensable physiologies such as those involved in ion and water balance are perhaps nowhere better studied than in fish models such as rainbow trout, and provide a wealth of information regarding the mechanisms involved in wholly new environments and organismal contexts. Rainbow trout remain one of the longest standing model organisms for a battery of amply justified reasons, and they will almost certainly remain a dominant model as improved methodologies, widespread whole-genome sequencing, and molecular techniques provide ever greater resolution of vertebrate biology and evolution.

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