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RESEARCH ARTICLE

Muscle growth in teleost fish is regulated by factors utilizing the activin II B receptor

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SUMMARY

The activin type IIB receptor (Acvr2b) is the cell surface receptor for multiple transforming growth factor β (TGF-β) superfamily ligands, several of which regulate muscle growth in mammals. To investigate the role of the Acvr2b signaling pathway in the growth and development of skeletal muscle in teleost fish, transgenic rainbow trout (RBT; *Oncorhynchus mykiss*) expressing a truncated form of the *acvr2b-2a* (*acvr2b*$^{\Delta}$) in muscle tissue were produced. High levels of *acvr2b*$^{\Delta}$ expression were detected in the majority of P1 transgenic fish. Transgenic P1 trout developed enhanced, localized musculature in both the epaxial and hypaxial regions (dubbed 'six pack'). The F1 transgenic offspring did not exhibit localized muscle growth, but rather developed a uniform body morphology with greater girth, condition factor and increased muscle fiber hypertrophy. There was a high degree of variation in the mass of both P1 and F1 transgenic fish, with several fish of each generation exhibiting enhanced growth compared with other transgenic and control siblings. The 'six pack' phenotype observed in P1 transgenic RBT overexpressing *acvr2b*$^{\Delta}$ and the presence of F1 individuals with altered muscle morphology provides compelling evidence for the importance of TGF-β signaling molecules in regulating muscle growth in teleost fish.

Key words: myostatin, trout, TGF-β, activin receptor IIB, muscle, transgenic.

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INTRODUCTION

The transforming growth factor β (TGF-β) superfamily of cytokines is essential for the regulation of numerous biological processes. Skeletal muscle growth is negatively regulated by several TGF-β molecules, including activin A and myostatin, which signal through the activin type I and type II serine/threonine kinase receptors (Link and Nishi, 1997; Lee et al., 2005; Lee et al., 2012). Activation of type II receptors by ligand binding causes hyperphosphorylation of serine residues on the glycine/serine-rich domain of type I receptors (Attisano et al., 1996), which subsequently propagates TGF-β signals by activation of the SMAD signaling cascade (Attisano and Wrana, 2002). While the activin type II receptors (A and B) are utilized by numerous TGF-β ligands, signaling specificity is maintained through combinations of SMAD and type I receptor activation (Feng and Derynck, 2005). The interaction between activin type II and type I receptors occurs through the intracellular serine/threonine kinase domains of each receptor and truncation of the type II kinase domain has been shown to inhibit signaling (Tsuchida et al., 2008). Overexpression of a truncated activin receptor IIB (ACVR2B) or supplementation with a soluble receptor is now commonly used to inhibit signaling through the dominant negative inhibition of endogenous ACVR2B (Wang and McPherron, 2012; Lee et al., 2012).

Myostatin, a ligand of the ACVR2B, is a key regulator of skeletal muscle growth in mammals. Deletion or inhibition of myostatin, through gene knockout (McPherron et al., 1997) or overexpression of inhibitors (Lee and McPherron, 2001), markedly increases muscle mass in mice. Similarly, 'double-muscled' phenotypes in cattle and whippets result from natural myostatin mutations (Kambadur et al., 1997; McPherron and Lee, 1997; Mosher et al., 2007). Myostatin, however, is not the only ACVR2B ligand required for regulating mammalian muscle growth. This is supported by the fact that injection of a soluble form of the ACVR2B or overexpression of follistatin, both of which inhibit several TGF-β molecules, increases muscle mass in myostatin-null mice (Lee et al., 2005; Lee, 2007). In addition to myostatin, activin A and GDF-11 have a similar affinity for the extracellular domain of ACVR2B (Sako et al., 2010) and have all been found to regulate myoblast differentiation *in vitro* (Souza et al., 2008).

Currently, little is known about the mechanisms regulating muscle growth in teleost fish, despite the fact that teleosts encompass almost half of all known vertebrate species (Zou and Jiang, 2008). The myostatin peptide sequence is highly conserved among fish and terrestrial vertebrates (Pie and Alvares, 2006); however, mammals possess a single form of myostatin found predominantly in muscle tissue, whereas multiple forms are differentially expressed throughout most of the tissues in teleost fish (Østbye et al., 2001; Rescan et al., 2001; Garikipati et al., 2006). Two forms of the *acvr2b* have recently been discovered in sea bream (*Sparus aurata*, Linnaeus 1758), with several other teleost species being identified as having multiple forms through bioinformatics resources (Funkenstein et al., 2012). The presence of multiple genes is common among teleost species because of a well-characterized genome duplication that occurred early in teleost evolution with a second gene duplication event occurring in the salmonid lineage.

Sea bream follistatin and the myostatin prodomain inhibit myostatin *in vitro*, suggesting that teleost myostatin is regulated by mechanisms similar to that of mammals (Rebhan and Funkenstein,

2008). *In vitro* studies have also demonstrated that the extracellular domain of sea bream Acvr2b-1 is capable of inhibiting myostatin signaling (Funkenstein et al., 2012). Small increases in muscle mass have been reported in homozygous transgenic zebrafish [*Danio rerio* (Hamilton 1822)] by overexpression of the myostatin pro-domain (Xu et al., 2003; Lee et al., 2009) and by myostatin RNAi (Lee et al., 2009). Myostatin-deficient medaka [*Oryzias latipes* (Temminck and Schlegel 1846)] also exhibit enhanced muscle growth (Chisada et al., 2011). Zebrafish and medaka, however, exhibit determinate growth and are restricted to minor increases in muscle mass (Biga and Meyer, 2009). An increase in mass has been reported for fish species capable of indeterminate growth (i.e. non-growth limited) following injection or submersion in a bath of a soluble form of the Acvr2b (Carpio et al., 2009) or myostatin pro-domain (Lee et al., 2010a); however, this research was limited to only larval and early juvenile life stages. In the research presented here, transgenic trout overexpressing a truncated *acvr2b-2a* (*acvr2b*$^{\Delta}$) were produced to investigate the involvement of Acvr2b signaling in the long-term regulation of muscle mass in a teleost species with indeterminate growth.

MATERIALS AND METHODS

Cloning RBT *acvr2b-2a*

Total RNA was extracted from white muscle (fast twitch) tissue of juvenile rainbow trout [RBT; *Oncorhynchus mykiss* (Walbaum 1792)] and adult zebrafish using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA), and cDNA was synthesized using oligo(dT)$_{18}$ primers (New England Biolabs, Ipswich, MA, USA) and Moloney murine leukemia virus reverse transcriptase (New England Biolabs). The complete RBT *acvr2b-2a* coding sequence (CDS) was amplified by PCR using degenerate primers (Integrated DNA Technologies, Coralville, IA, USA) designed to conserved regions of the zebrafish (GenBank accession no. AF069500) and grass carp [*Ctenopharyngodon idella* (Valenciennes 1844), FJ198047] *acvr2b* mRNA sequences (Table 1).

DNA construct

A truncated *acvr2b-2a* construct [*Tg(RA.Mlc:acvr2b*$^{\Delta}$*)*] was prepared using a 528 bp fragment of the 5′ end of the zebrafish *acvr2b-2a* CDS, which included 31 bp of the 5′ untranslated region, the extracellular and transmembrane domains, and a residual 17 bp of the kinase domain. The zebrafish *acvr2b-2a* sequence was used for production of transgenic RBT because of its detailed molecular characterization (Garg et al., 1999) and conservation to RBT *acvr2b-2a* (89% amino acid conservation of the extracellular domain). Using the zebrafish *acvr2b-2a* sequence also enabled differentiation from the native RBT receptor transcript for precise quantification of transgene expression levels. The *acvr2b*$^{\Delta}$ was cloned into a skeletal muscle-specific expression vector containing a rat myosin light chain (*RA.Mlc*) promoter and enhancer and an SV40 polyadenylation sequence, generously provided by Dr Shao Jun Du of the University of Maryland (Rosenthal et al., 1989; Xu et al., 2003; Medeiros et al., 2009). This construct was separated from the vector backbone by NotI (New England Biolabs) restriction digestion and the linear construct was purified by electrophoresis through a 1% agarose gel and quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Newark, DE, USA). The *Tg(RA.Mlc:acvr2b*$^{\Delta}$*)* construct was sequenced in both directions at the University of Rhode Island Genomics and Sequencing Center (URI GSC) to verify sequence and insert orientation.

Microinjection

Rainbow trout ova (Arlee strain; Ennis National Fish Hatchery, Ennis, MT, USA) were fertilized 30 min to 3 h prior to use and maintained at 4°C in RBT isotonic saline (9.04 g l^{-1} NaCl, 0.24 g l^{-1} KCl, 0.34 g l^{-1} $CaCl_2$) to prevent hardening of the chorion and to slow development. Ova were microinjected through the micropyle with 1 million copies of linearized construct in a total volume of 18 nl as previously described (Medeiros et al., 2009).

Husbandry

After microinjection, ova were transferred to flow through vertical tray incubators (MariSource, Fife, WA, USA). Following yolk sac absorption, trout were transferred to 0.27 m^3 tanks supplied with single pass water ranging seasonally from 4 to 16°C and exposed to a simulated natural photoperiod. The fish were maintained at low densities and fed seven times daily with a commercially formulated feed (Corey Feed Mills, Fredericton, NB, Canada). After screening for the presence of the construct, a random sample of non-transgenic (control) fish were marked by excision of the adipose fin and raised communally with transgenic fish to eliminate environmental variability (i.e. tank effect). All RBT were implanted with a passive integrated transponder (PIT) tag (Biomark, Boise, ID, USA) and transferred to 2 m diameter (1.7 m^3 volume) flow through tanks once they attained a mass of 15 g. Juvenile trout were fed to satiation three times daily with commercial trout feed (Silver Cup, Murray, UT, USA) and adult RBT were provided a broodstock feed (Aquabrood; Corey Feed Mills) 6 months prior to sexual maturation to enhance

Table 1. Primer sets used in the production, screening and qRT-PCR of *acvr2b*$^{\Delta}$ transgenic trout and those used in discovery of the rainbow trout (RBT) *acvr2b-2a* gene

Primer	Orientation	Sequence (5′–3′)
ZFacvr2b-F	Fwd	GCT CCT CCG GGT TTA TTT TC
ZFacvr2b-R	Rev	GAG GGC ACT CTA GGC TTG TG
Mylc 5	Fwd	CAC CAC TGC TCT TCC AAG TGT CA
acvr2b R6	Rev	TGT CCG GCA GGT GTG TAA ATC TCT
RBT acvr2b F1	Fwd	ATT ARA GGA ATA TGT TCG CTT C
RBT acvr2b R342	Rev	CGT TGA CAT CAG GCA GGT A
RBT acvr2b F93	Fwd	GCA CTG CTA CGC CTC VTG G
RBT acvr2b R1141	Rev	CCT CCT CAA ACG GCA GCA TGT AC
RBT acvr2b F970	Fwd	TAA GAA TGT GAT GCT GCG GAC
RBT acvr2b R1604	Rev	GTT CAG ATG CTG GAC TCT TTG G
qRT-PCR acvr2b F230	Fwd	GCT GGC TCG ACG ACT TCA ACT
qRT-PCR acvr2b R4	Rev	CAT AGG GTG GCT TGT GGT GGC G
qRT-PCR gapdh F	Fwd	ACC ACT ACA ACC CAA TCA ACA GCA A
qRT-PCR gapdh R	Rev	TCG ATG AAG GGA TCG TTG ATG GC

ova quality. Fish that reached maturity at 2 years of age were manually spawned using established husbandry practices (Piper et al., 1982) and the gametes from transgenic trout were used to produce F1 families. F1 offspring were raised in conditions identical to those of the parental generation (i.e. communal tanks and adipose fin clip for non-transgenic fish) but were not PIT tagged for identification of individuals. During handling and sampling, fish were anesthetized in a solution of 0.075 g l^{-1} tricaine methanesulfonate (Argent Chemical Laboratories, Redmond, WA, USA) to reduce stress and facilitate handling. All protocols involving animals were approved by the University of Rhode Island Institutional Animal Care and Use Committee (AN01-04-031 and AN08-02-012).

Transgenic screening

Juvenile RBT were screened for integration of the construct at a body mass of ~2 g using an established protocol (Medeiros et al., 2009). Briefly, a small quantity of caudal fin was excised and assayed by PCR with a vector-specific forward primer and an *acvr2b*$^{\Delta}$-specific reverse primer (Table 1). Transgenic fish were identified by the presence of an amplicon after electrophoresis of the PCR products through a 1% agarose gel.

Gene expression

White muscle tissue was excised from P1 juveniles by a 4 mm biopsy (Premier Medical Products Company, Plymouth Meeting, PA, USA) adjacent to the dorsal fin or by lethal sampling in the F1 generation. Residual skin was removed and 20–25 mg of muscle was flash frozen in a dry-ice–ethanol bath. The tissue was homogenized with a 5 mm stainless steel bead in a chaotropic solution using a TissueLyser (Qiagen, Valencia, CA, USA) at 25 Hz for 5 min. RNA was isolated from the tissue homogenates after aqueous phase separation using 96-well RNeasy plates (Qiagen). To eliminate the potential of DNA contamination, samples were digested on the column with 80 µl of DNase I (0.34 Kunitz µl^{-1}, Qiagen) for 15 min. Purified RNA was eluted in RNase-free molecular grade water followed by short-term storage at −80°C.

Quantitative reverse transcription PCR (qRT-PCR) with SYBR Green fluorescence (Brilliant SYBR Green qRT-PCR Master Mix, 1 step, Stratagene, La Jolla, CA, USA) was used to verify *acvr2b*$^{\Delta}$ expression. The qRT-PCR reaction was conducted using 50 ng of purified total RNA under the following conditions: 50°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 30 s and 65°C for 1.5 min. A dissociation curve was generated to ensure a single amplicon was produced, by denaturing each amplified sample at 95°C for 1 min, followed by 55°C for 30 s, and increasing the temperature by 0.5°C per 30 s cycle for 80 cycles. All qRT-PCR samples were assayed in triplicate alongside a duplicate six-point standard curve made from purified PCR product. Triplicate samples lacking reverse transcriptase enzyme or template were assayed to verify the absence of DNA or reagent contamination. Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) expression was also quantified for each sample to normalize expression data. Analysis of *gapdh* samples was performed with an annealing temperature of 63°C, in triplicate with appropriate standard curve and control samples similar to the *acvr2b*$^{\Delta}$ qRT-PCR assay. All reactions were performed on a MX4000 Real Time PCR system (Stratagene) and amplicons from both qRT-PCR reactions and respective standard products were sequenced at the URI GSC.

Growth and phenotypic characterization

The growth of each P1 transgenic and non-transgenic sibling control fish was measured five times between 9 and 15 months of age by obtaining a digital image and mass to the nearest 0.1 g. Length measurements were obtained from photographs using Image-Pro Plus version 5.0.1 (MediaCybernetics, Bethesda, MD, USA) after calibrating pixels per centimeter at two locations on the image. Length was measured to the nearest 0.01 cm from the tip of the snout to the center, posterior edge of the caudal peduncle. The P1 generation was randomly divided into cohorts 1 (N=20 transgenic fish) and 2 (N=10 transgenic fish) to maintain low densities. The growth of each cohort was analyzed separately. Condition factor and specific growth rate were calculated for each individual transgenic and control fish. Condition factor, a ratio of fish mass to length, was calculated using the formula $(M/L^3)\times100$, where M is mass in grams and L is length in centimeters. Specific growth rate is defined as the percent of body mass gained per day over a defined period and is calculated by the formula: {[ln(final mass)–ln(initial mass)]×100}/total days between measurements.

To examine the growth characteristics of the F1 generation, fork length (cm) and mass (g) of *acvr2b*$^{\Delta}$ trout were measured six times between 5 and 10 months of age. Nine transgenic fish from line *Tg(RA.Mlc:acvr2b*$^{\Delta}$*)aa*26-24 were raised in a 1.6 m^3 circular tank with a random group of 19 non-transgenic siblings (controls). The condition factor for F1 transgenic and control fish was calculated at each growth inventory using length and mass measurements.

Muscle histology

Muscle histology was examined in *Tg(RA.Mlc:acvr2b*$^{\Delta}$*)aa*26-24 F1 offspring at 4 months post-hatch (6.6±0.4 g, 7.4±1.4 cm). Seven transgenic and control fish were euthanized with an overdose of tricanine methanesulfonate followed by measurement of mass and length. Two 5×5 mm cubes were excised from the epaxial section of the ‘steak’ just anterior to the dorsal fin and oriented to allow for a cross-sectional cut. Tissue was frozen in cryosectioning embedding medium (Tissue tek, Thermo Fisher Scientific) for 20 s in isopentane chilled in a bath of liquid nitrogen. Muscle fiber number, diameter and the area of cross-sectional muscle tissue were analyzed as previously described (Medeiros et al., 2009). The total cross-sectional muscle surface area and subsample muscle fiber number were used to extrapolate the total fiber number per cross-section.

Data analysis

Statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC, USA). Mass, length, condition factor and specific growth rate of P1 *acvr2b*$^{\Delta}$ transgenic and sibling control fish were compared using a two-way ANOVA with repeated measures on one factor after testing for sphericity. An independent samples t-test, with or without the assumption of equal variances between groups, was used to analyze F1 cross-sectional area, total fiber number, muscle fiber diameter and unit fiber number, and for the growth studies of the F1 generation. Pearson correlations were calculated comparing *acvr2b*$^{\Delta}$ expression levels with the mass and length of P1 transgenic fish. Values are reported in the text as means ± s.e.m. Differences were considered significant at P<0.05.

RESULTS

Rainbow trout *acvr2b-2a* CDS

The full-length CDS of RBT *acvr2b-2a* (GenBank accession no. HM143891) possessed a high degree of conservation with other teleost *acvr2b* sequences including: Atlantic salmon (94%, *Salmo salar*, Linnaeus 1758, EF063143.1), grass carp (83%, *C. idella*, FJ198047.1), zebrafish (82%, *D. rerio*, NM_131210.2), gilthead sea bream (82%, *S. aurata*, JF906099) and goldfish [80%, *Carassius auratus* (Linnaeus 1758), AF001406.1]. The mRNA sequences of chicken [*Gallus gallus*

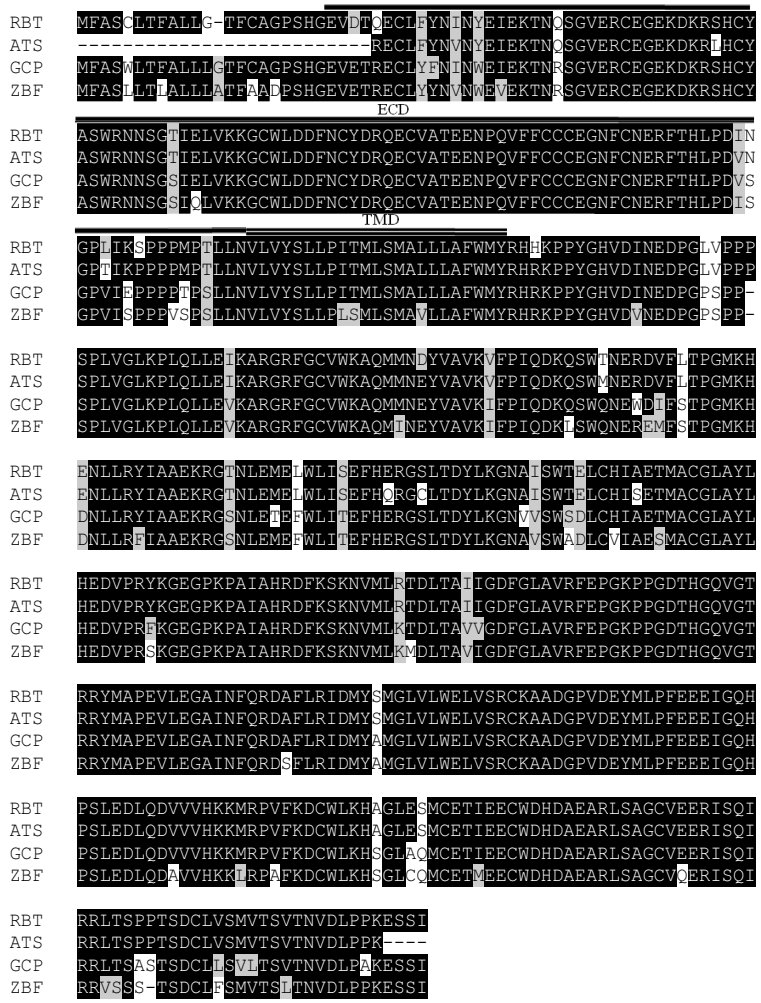

Fig. 1. Rainbow trout (RBT) Acvr2b-2a deduced amino acid sequence (ADJ19047) aligned with protein sequences from Atlantic salmon (ATS; ABK54368), grass carp (GCP; ACI23559) and zebrafish (ZBF; AAD19844). The extracellular and transmembrane domains are identified with single and double over-lines, respectively. Conserved (black), similar (gray) and unique (white) amino acids are highlighted.

(Linnaeus 1758), NM_204317.1] and human (*Homo sapiens*, Linnaeus 1758, BC099642.4) *ACVR2B* were 76% similar to RBT *acvr2b-2a*. The deduced amino acid sequence of the extracellular domain of RBT Acvr2b-2a was 89% identical to that of zebrafish (Fig. 1), and all 10 cysteine residues conserved in the extracellular domain of other Acvr2b proteins (Garg et al., 1999) were present.

Rate of transgenesis and maturation

A total of 1581 fertilized eggs were microinjected with the *Tg(RA.Mlc:acvr2b*$^{\Delta}$*)* DNA construct. Of the 292 surviving juvenile trout, 48 integrated the DNA construct (16.4%). Of these, only 18% of the P1 transgenic females spawned at 2 years of age, despite being of sufficient size (2–4 kg) to attain sexual maturation; 50% of control females spawned. Examination of three moribund females post-spawning season revealed developing reproductive tissue in two individuals with no signs of oocyte development in the third. At 3 years of age, the remaining control and *acvr2b*$^{\Delta}$ females matured. There was no difference in the time of maturation for control and *acvr2b*$^{\Delta}$ males. Mendelian inheritance was not observed in the majority of *acvr2b*$^{\Delta}$ × *acvr2b*$^{\Delta}$ F1 families, suggesting that there was substantial germline transgene mosaisicm in P1 founders.

acvr2b$^{\Delta}$ expression

Absolute qRT-PCR analysis detected *acvr2b*$^{\Delta}$ expression in the muscle tissue of most P1 transgenic RBT, with expression varying widely among individuals (0–7,657,000 transcripts). The majority of transgenic fish (69%) produced in excess of 1,000,000 copies of *acvr2b*$^{\Delta}$ per 50 ng of total RNA. No *acvr2b*$^{\Delta}$ transcript was detected in 12 fish that assayed negative for the presence of the construct in gDNA. The expression of *gapdh* did not vary between transgenic (19,943±1919 transcripts) and control (19,411±1038 transcripts) fish. There was no correlation between the mass or length of P1 transgenics and *acvr2b*$^{\Delta}$ transcript levels (mass R^2=0.134, length R^2=0.163). The highest transcript levels were observed in two of the smallest individuals, which exhibited an abnormally short, compact body morphology (7,657,000 and 6,025,000 transcripts), whereas the two largest fish had moderate levels of *acvr2b*$^{\Delta}$ expression (2,015,000 and 1,507,000 transcripts). Transgene expression was detected in all assayed F1 transgenic lines, with expression ranging between 100,000 and 1,000,000 copies per 50 ng of RNA, depending on the line (data not shown).

Transgenic P1 phenotype

A phenotype characterized by enhanced muscling, with numerous individuals having both well-defined hypaxial regions as well as large masses of epaxial muscle, was observed in P1 *Tg(RA.Mlc:acvr2b*$^{\Delta}$*)* transgenic fish (Fig. 2A). In several individuals, examination of the interior abdominal wall revealed protrusions of muscle between the hemapophysial ribs corresponding to locations of external ridges. The degree and location of muscling varied among transgenic individuals, with some exhibiting muscle asymmetry (Fig. 2B). No additional morphological anomalies were observed in

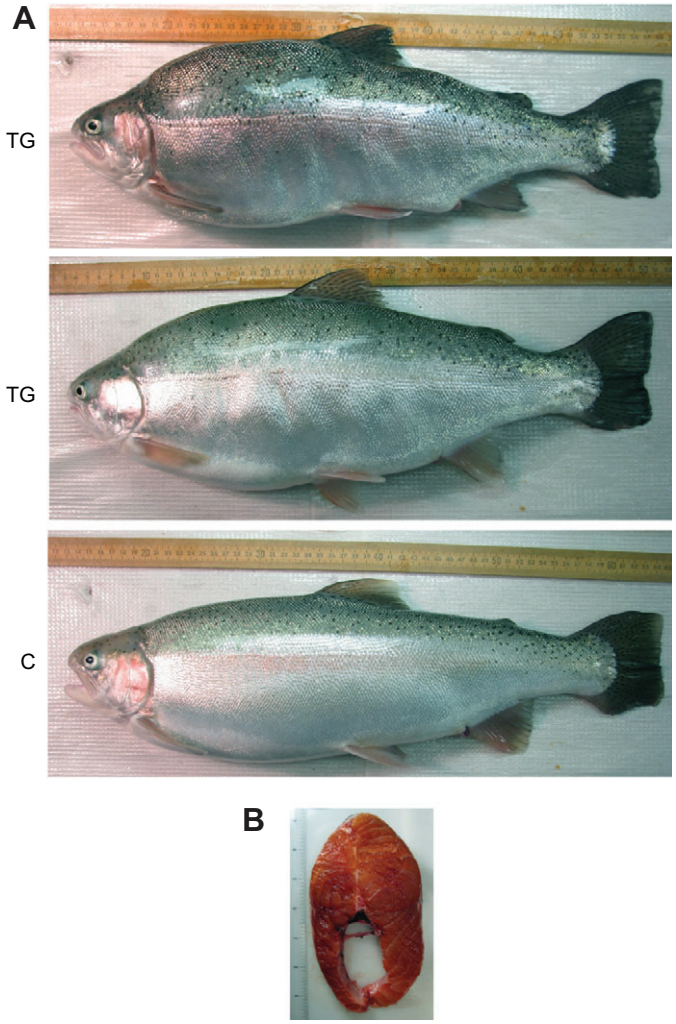

Fig. 2. Localized muscling exhibited by the P1 founder generation of rainbow trout. (A) Morphology of 2-year-old *acvr2b*$^{\Delta}$ transgenic (TG) and control (C) broodstock. P1 transgenic individuals exhibit localized muscling in both the abdominal and epaxial regions of the musculature. (B) Cross-section of a P1 transgenic fish reveals asymmetrical skeletal muscle growth.

P1 transgenic fish and all internal organs appeared normal. Enhanced muscling did not appear to affect swimming or feeding activity.

P1 transgenic growth

There were no significant differences between the mean mass and length of P1 transgenic and control trout when analyzed between 9 and 15 months of age. Transgenic (TG) fish had significantly higher condition factor values compared with control fish (cohort 1: TG 2.15±0.051, control 1.83±0.051; cohort 2: TG 1.93±0.045, control 1.75±0.032) throughout the study, supporting the observation that most P1 transgenic individuals had a compact body morphology (Fig. 2A). The increased condition factor was reflective of the fact that despite the lack of significant differences in mass and length, transgenic fish tended to be heavier when compared with control fish of similar length, as shown by the mass to length distribution of both cohorts (Fig. 3). At 15 months of age, one transgenic fish from cohort 2 was 65% heavier than the largest control fish (1004 *versus* 655 g) with one of the highest condition factor values (Fig. 3). There was no significant difference in the specific growth rate between P1 transgenics and controls.

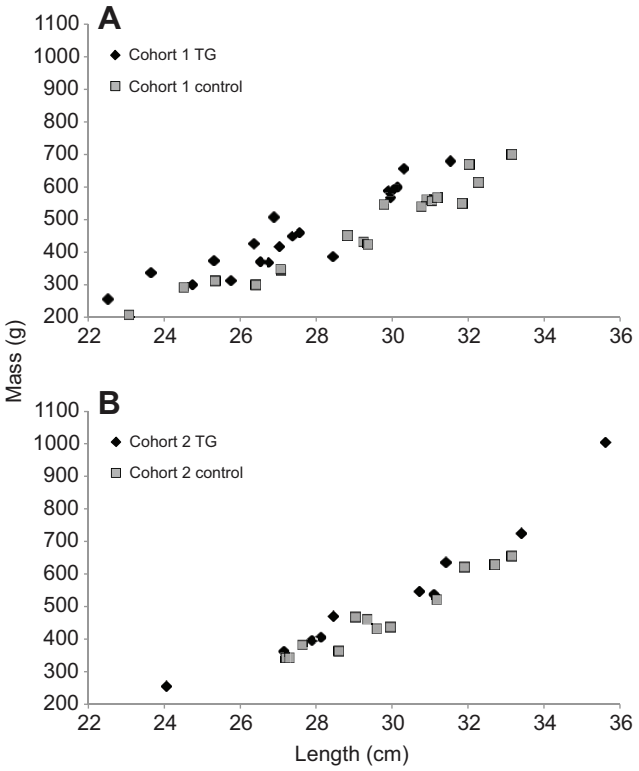

Fig. 3. Size distribution of P1 transgenic rainbow trout at 15 months of age. Mass and length measurements are plotted for individual transgenic (TG; black diamond) and control (gray square) fish from cohort 1 (A) and cohort 2 (B).

F1 morphology and muscle histology

F1 transgenic offspring did not possess the 'six pack' phenotype observed in the P1 generation, but most individuals exhibited a distinct compact body morphology that was readily apparent (Fig. 4). The number of muscle fibers per 0.254 mm^2 area of muscle tissue

Fig. 4. Morphology of rainbow trout F1 transgenic offspring. Transgenic (TG) F1 fish exhibited a compact body morphology compared with control fish (C) without the presence of localized muscle growth.

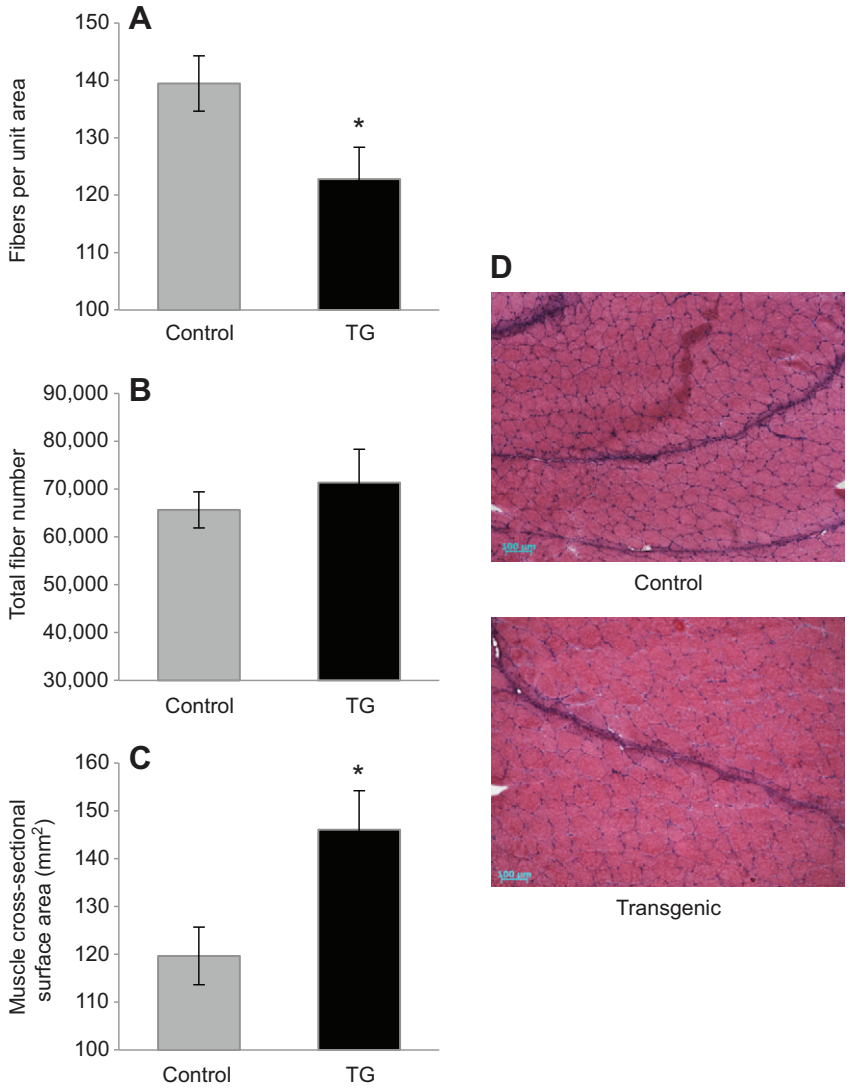

Fig. 5. Mean (A) fiber number per 0.254 mm^2 unit area, (B) total fiber number per cross-section and (C) cross-sectional muscle surface area for 4-month-old transgenic (black) and control (gray) rainbow trout. (D) Muscle histology sections from representative control and transgenic trout indicating muscular hypertrophy in transgenic fish. Asterisks indicate values statistically different from control fish.

in 4-month-old transgenic individuals (122.8±5.6) was significantly lower than in control fish (139.5±4.8; Fig. 5A,D), while transgenic muscle fiber diameter (41.05±0.84 μm) was significantly larger compared with controls (38.22±0.76 μm). Despite differences in the size of muscle fibers, the overall number of muscle fibers per cross-sectional area did not differ between groups (TG 71,405±7010; control 67,771±3415; Fig. 5B) because of a significant increase in the cross-sectional surface area of transgenic fish (146.1±8.1 mm^2) compared with control fish (120.3±5.3 mm^2; Fig. 5C).

F1 transgenic growth

The growth characteristics of F1 siblings from one P1 *acvr2b*$^{\Delta}$ × *acvr2b*$^{\Delta}$ cross was assessed between 5 and 10 months post-hatch [line *Tg(RA.Mlc:acvr2b*$^{\Delta}$*)aa*26-24]. No significant difference was observed between the mean mass of *Tg(RA.Mlc:acvr2b*$^{\Delta}$*)aa*26-24 transgenic and control fish populations, but a distinct upper mode of transgenic fish weighed significantly more than the average control fish throughout the study (Fig. 6A). At 10 months of age, the upper mode of *Tg(RA.Mlc:acvr2b*$^{\Delta}$*)aa*26-24 transgenic fish was 31% heavier than controls (upper mode 621.2±58.9 g, control 475.1±20.9 g), with the largest transgenic weighing 55 and 13% more than the average and largest control fish, respectively (Fig. 6A). No difference in length was detected between the upper mode of transgenic and control fish, but the mean length of the total transgenic population was significantly less than controls at 10 months of age (Fig. 6B). The *Tg(RA.Mlc:acvr2b*$^{\Delta}$*)aa*26-24 transgenic fish had significantly higher condition factor values throughout the study (Fig. 6C).

DISCUSSION

Expression of *acvr2b*$^{\Delta}$ in P1 RBT stimulated enhanced localized muscling in the hypaxial and epaxial regions of the musculature. This is similar to what we have observed in RBT overexpressing follistatin (Medeiros et al., 2009), a known antagonist of several TGF-β ligands (Welt et al., 2002; Gilson et al., 2009) that utilize the *acvr2b*. This unique phenotype has now been documented in two strains of transgenic RBT with different inhibitors of TGF-β signaling (i.e. *acvr2b*$^{\Delta}$ and follistatin), providing further evidence that ligands of the TGF-β superfamily regulate muscle growth in teleost fish. The phenotype is unique in that distinct localized regions of muscle growth develop in the relatively uniform morphology of the trout axial musculature. This may be a result of global muscle growth without a concurrent increase in length, because transgene expression was driven by a muscle-specific promoter. Alternatively, the phenotype may be related to somatic mosaicism in P1 transgenic fish, which could cause differential TGF-β inhibition in the musculature, resulting in localized regions of muscle growth. The lack of the ‘six pack’ phenotype in the F1 generation suggests that

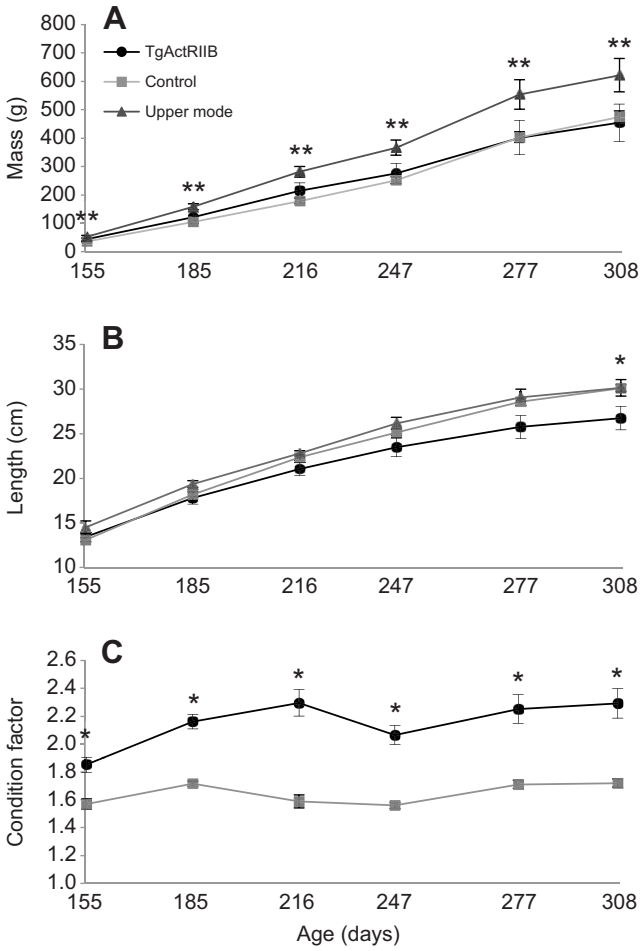

Fig. 6. Growth characteristics of rainbow trout F1 transgenic line *Tg(RA.Mlc:acvr2b$^{\Delta}$)aa*26-24. Growth pattern in (A) mass, (B) length and (C) condition factor of the average transgenic (black circle), control (gray square) and the upper transgenic mode (dark gray triangle) of fish between 5 and 10 months of age. Asterisks indicate statistical significance of the average transgenic fish (*) or the upper mode of transgenic fish only (**) compared with controls.

somatic mosaicism was likely the cause of localized muscling in the P1 generation. Late-stage genomic integration after construct microinjection is common in both fish and mammalian P1 transgenic organisms, and can lead to somatic and germ tissue mosaicism (Stuart et al., 1990; Gross et al., 1992; Whitelaw et al., 1993; Rahman et al., 2000). Somatic mosaicism has been reported to occur in up to 62% of transgenic mice produced by microinjection (Whitelaw et al., 1993), with only 10–20% of the cells in a given tissue integrating the plasmid construct (Palmiter et al., 1984).

In mammals, a number of TGF-β ligands including myostatin, activin, GDF-11, nodal and several bone morphogenetic proteins are known to signal through the ACVR2B (Tsuchida et al., 2008). Activin A is suggested to work in concert with myostatin to regulate muscle growth in mammals (Gilson et al., 2009; Lee et al., 2010), and may have a similar role in teleost fish. However, it remains unclear which TGF-β molecules are primarily responsible for the enhanced muscling observed with *acvr2b*$^{\Delta}$ expression in RBT.

A range of growth rates was observed in P1 *acvr2b*$^{\Delta}$ transgenic fish, with several individuals growing to substantial size, but overall the transgenic organisms were not significantly larger than control fish. Size variation is common in P1 transgenic fish overexpressing growth hormone and has been observed in P1 transgenic coho salmon [*Oncorhynchus kisutch* (Walbaum 1792)], common carp [*Cyprinus carpio* (Linnaeus 1758)] and northern pike [*Esox lucius* (Linnaeus 1758)] (Gross et al., 1992; Devlin et al., 1995; Wang et al., 2001). Only 8.7% of the growth hormone transgenic carp exhibited enhanced growth, with many fish having reduced growth (Wang et al., 2001). As a result of random genome integration, variation in the number of integrated copies and concatemerization of the DNA construct, each founder possesses a unique transgenic genotype (Culp et al., 1991; Uh et al., 2006), which is reflected in the observed morphological variation.

Although localized muscle growth was not present in the F1 generation, an overall compact body morphology developed that was readily apparent and led to a significantly higher condition factor in transgenic individuals. The phenotype was present in the majority of F1 transgenic fish regardless of size, with the largest individuals exhibiting some of the highest condition factors. Increased condition factor was also observed in transgenic RBT overexpressing follistatin (Medeiros et al., 2009) and in mice with the *Cmpt* myostatin mutation, which develop enhanced muscling and a short, compact body morphology (Varga et al., 1997). It is currently unclear as to the mechanism behind the reduced length exhibited by *acvr2b*$^{\Delta}$ transgenic fish. It is possible that *acvr2b*$^{\Delta}$ expression may have unknown side effects that could influence TGF-β signaling in adjacent tissues. This may also explain the delayed maturation observed in many P1 transgenic females. More research is needed to fully understand the functional significance of these phenotypic observations.

Transgenic F1 individuals at 4 months post-hatch increased muscle mass primarly through hypertrophy. This differed from transgenic trout overexpressing follistatin, in which muscle fiber hyperplasia was the primary mechanism for the observed increase in muscle mass (Medeiros et al., 2009). There is currently little consensus on the specific mechanism by which TGF-β ligands regulate muscle growth. Myostatin-null medaka increase muscle mass by hyperplasia, then switch to hypertrophy in adulthood (Chisada et al., 2011). While both hypertrophy and hyperplasia of muscle fibers have been documented with TGF-β and myostatin inhibition in mice (Lee and McPherron, 2001), the primary mechanism in mammals appears to be hypertrophy (Amthor et al., 2009). Recent mammalian evidence suggests that myostatin and activin A regulate muscle hypertrophy by directly targeting muscle fibers with little contribution from satellite cells (Wang and McPherron, 2012). TGF-β inhibition from follistatin and soluble ACRV2B has been shown to increase muscle fiber hypertrophy in mice with minimal addition of new myonuclei to existing myofibers. These treatments also increase muscle fiber hypertrophy in mice with inactivated satellite cells (Wang and McPherron, 2012; Lee et al., 2012). Targeted deletion of *Acrv2b* in mouse muscle fibers also induces muscle fiber hypertrophy, supporting the idea that satellite cells are not required for hypertrophic growth (Lee et al., 2012). Transgenic *acvr2b*$^{\Delta}$ likely utilizes a similar mechanism to increase muscle fiber hypertrophy as the truncated protein should only be expressed in fully differentiated muscle fibers. Transgenic fish overexpressing follistatin may exhibit a slightly different mechanism of TGF-β inhibition because the soluble protein is not restricted to muscle fibers. It is also possible that differences observed in *acvr2b*$^{\Delta}$ and follistatin transgenic trout are related to the size or age of the fish or to differential regulation of distinct TGF-β ligands. The follistatin-overexpressing transgenic fish were analyzed at a mass of ~195 g and a length of 19 cm (Medeiros et al., 2009), whereas

the F1 *acvr2b*$^{\Delta}$ fish were analyzed at a significantly smaller size (6.6±0.4 g, 7.4±1.4 cm).

Variation in the growth of the F1 generation limited direct conclusions on the impact of *acvr2b*$^{\Delta}$ expression on the overall growth of transgenic trout, despite the fact that several transgenic individuals from both F1 lines exhibited increased muscle growth. Variation in F1 transgenic fish is not uncommon. Significant variation has been reported in the F1 generation of transgenic growth hormone enhanced common carp (Wang et al., 2001) and arctic char, *Salvelinus alpinus* (Linnaeus 1758) (Pitkänen et al., 2001). Similarly, two transgenic lines exhibiting dramatically different phenotypes have also been reported from the F1 offspring of a single transgenic mouse overexpressing the myostatin pro-peptide domain under the control of the same *RA.Mlc* promoter (Lee and McPherron, 2001) used to produce *Tg(RA.Mlc:acvr2b*$^{\Delta}$*)* transgenics. Further variation was added to the F1 generation of *Tg(RA.Mlc:acvr2b*$^{\Delta}$*)aa*26-24 transgenic trout by crossing transgenic individuals. This was done to maximize the number of transgenic genotypes in the F1 generation given constraints on rearing significant numbers of large transgenic fish, which required 2 years to reach maturity. Despite the lack of conclusive growth data, the morphology exhibited by this model organism provides compelling *in vivo* evidence that ligands of the Acvr2b-2a regulate muscle growth and morphology in teleost fish.

LIST OF ABBREVIATIONS

Acvr2b	activin receptor 2B
acvr2b$^{\Delta}$	truncated activin type IIB receptor
CDS	coding sequence
F1	first generation offspring
gapdh	glyceraldehyde 3-phosphate dehydrogenase
P1	founder parental generation
PIT	passive integrated transponder
qRT-PCR	quantitative reverse transcription PCR
RBT	rainbow trout
TGF-β	transforming growth factor β

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AUTHOR CONTRIBUTIONS

M.P.P. conducted the majority of the research on the project and preparation of the manuscript. The research reported here was a major component of his dissertation. I.M.J. conducted specific experiments for the project and also assisted in preparation of the manuscript. T.M.B. is the PI and was responsible for conception of the research project, supervision of the research and preparation and revisions of the manuscript.

COMPETING INTERESTS

No competing interests declared.

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