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Effects of Myostatin Prodomains on the Reproduction of Rotifer *Brachionus rotundiformis*

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Myostatin (MSTN), a member of the transforming growth factor (TGF)-beta family, is a potent negative regulator of skeletal muscle growth and maintenance. The MSTN prodomain inhibits MSTN biological activity. The rotifer *Brachionus rotundiformis* is an excellent primary live feed for fish larvae in aquaculture; however, it is not known whether the rotifer expresses MSTN and the MSTN prodomain along with its activity. The objective of this study was to examine the effects of recombinant MSTN prodomains. Individual cultures of the rotifer *B. rotundiformis* were carried out to determine the effect of recombinant MSTN prodomains (pMALc2x-poMSTNpro and pMALc2x-sMSTNpro) on the pre-reproductive phase, reproductive phase, post-reproductive phase, offspring, lifespan, fecundity, and male ratio. In addition, a population culture of the rotifer was performed to confirm the effects of pMALc2x-poMSTNpro and pMALc2x-sMSTNpro on population growth. The results showed that the rotifer treated with pMALc2x-pMSTNpro had a reduced pre-reproductive phase at higher concentrations (1, 2, and 4 μg/ml) compared to the non-treated control group. Moreover, the pMALc2x-sMSTNpro treated rotifer effectively decreased the pre-reproductive phase at a lower concentration (0.25 μg/ml) compared to the pMALc2x-pMSTNpro treated and control group. Interestingly, pMALc2x-poMSTNpro and pMALc2x-sMSTNpro significantly increased the population of *B. rotundiformis*.

Key words : Myostatin, myostatin prodomain, rotifer, *B. rotundiformis*, reproduction

Introduction

Myostatin (MSTN), a member of the TGF (transforming growth factor)-beta family, is a potent negative regulator of skeletal muscle growth and maintenance [20] by suppressing proliferation and differentiation of myoblasts [18,32]. Mutations of MSTN gene have been reported to cause dramatically increased muscle mass in a variety of mammals, including cattle breeds [13,21,27], mice [29,37], sheep [5], dogs [22] and recently even in human [26]. These are suggests that the biological functions of MSTN are conserved in mammals.

Various studies indicate that MSTN forms a disulfide-linked dimer like many other members of the TGF-β superfamily [13,20,31]. The cleaved propeptide molecule is remains by non-covalently bound to the dimer mature domain, forming a latent complex and resulting in inhibition of the biological activity of MSTN by inhibiting MSTN binding to ActRIIB receptors [18,31]. The metalloproteinases of born morphogenetic proteins-1/tolloid (BMP-1TLD) activate MSTN by proteolytic cleavage of prodomain [33]. Study of the effect of MSTN prodomain has also shown that more than 70% of MSTN circulates in mouse serum as a latent complex containing MSTN prodomain, which maintains the C-terminal dimer in a latent, inactive state [12]. The importance of MSTN prodomain for mature MSTN activity was further demonstrated in transgenic mice over-expressing the MSTN prodomain, which showed a dramatic muscling phenotype with a 17-30% increase in body weight [35].

MSTN function is well documented in mammalian species, role of MSTN in fish and microalgae is less known. In contrast to mammals and chicken, fish possess two distinct MSTN genes with differential expression [24]. Moreover, although the mature MSTN in fish is 109 amino acids and its sequence is highly conserved compared to other vertebrates and fish species [14]. By contrast, MSTN prodomain varies between fish species in its length and its amino acid sequence [19,30]. MSTN not only inhibits skeletal muscle growth, but also plays important roles in maintenance of homeostatic tissue growth, osmolality regulation,

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and reproductive tissue function in fish [25]. A few recently studies, using transgenic zebrafish of overexpressing the MSTN prodomain [34], morpholino injections [3] and RNA interference [1] strongly suggest that MSTN inhibit muscle growth in fish as in mammals. Thus availability of fish prodomain is used for studies that elucidating the physiological role of MSTN in fish.

The invertebrate rotifer belongs to the Lophotrochozoa and used worldwide as the primary live feed for the initial larval stages of marine fish. They also have been used as models in ecotoxicology and population dynamics because of sensitivity against the chemical substance which introduced into aquatic environment, rapid population growth and easy culture [2,11,23,36]. The monogonont rotifer *Brachionus rotundiformis* is ability to reproduce both asexually and sexually. In asexual or amictic reproduction, amictic female produces amictic eggs that are diploid (2N) and hatch only into amictic females via ameiotic parthenogenesis, whereas in sexual or mictic reproduction, female produces haploid (N) eggs via meiosis. These eggs will develop into haploid males but if fertilized they will become diploid resting eggs that undergo dormancy, eventually hatching as amictic females.

In zooplankton rotifers, presence and function of MSTN is not known. Thus, this study was designed to investigate the effect of two fish species (*Paralichthys olivaceus* and *Sebastes schlegeli*) derived recombinant MSTN prodomains (pMALc2x-pMSTNpro and pMALc2x-sMSTNpro, respectively) on rotifer *B. rotundiformis* pre-reproductive phase, reproductive phase, post-reproductive phase, offspring, lifespan, fecundity and male ratio by individual culture. And flask culture of the rotifers performed to confirm the effects of pMALc2x-poMSTNpro and pMALc2x-sMSTNpro on its population growth.

Materials and Methods

Rotifers

The zooplankton rotifers used in these experiment are *Brachionus rotundiformis* (S type), Uljin strain, which were continuously cultured in the live food lab, Faculty of Marine Bioscience and Technology, Gangneung-Wonju National University, Korea. Rotifers were cultured in 15 psu seawater at 28°C under constant fluorescent illumination 1,000 lux. Freshwater Chlolella, C*hlolella vugaris* was fed to *B. rotundiformis* every two days for 7×10^6 cells/ml.

Recombinant protein expression and purification

Two fish species (*Paralichthys olivaceus* and *Sebastes schlegeli*) recombinant MSTN prodomains (pMALc2x-poMSTNpro and pMALc2x-sMSTNpro, respectively) were expressed and purified as previously described [16,17]. Purified recombinant MSTN prodomains were dialyzed in Tris-HCl (pH 7.0) and freeze dried. And it was stored at -80°C deep freezer.

Reproduction test

Recombinant MSTN prodomains were investigated the effect on reproduction of rotifer. Rotifers were pre-cultured to acquire enough population. Rotifers which having eggs were chosen from the batch culture population, transferred to 6-well culture plate containing 15 psu seawater and cultured at 28°C incubator without changing the medium. The hatched neonates (age<2 h) were used for the individual culture. Individual neonates were transferred into 24-well culture plate containing 1 ml of 15 psu seawater and recombinant MSTN prodomains. Tested concentrations of recombinant MSTN prodomains were 0 (control), 0.25, 0.50, 0.75, 1, 2, and 4 μg/ml. Because Tris-HCl (pH 7.0) buffer was used as a solvent, solvent control was included in all experiments. After 12 h, treated of recombinant MSTN prodomains the rotifers were observed every hour and recorded the time of appearance the first egg (pre-reproductive phase). Eggs hatched, offspring were transferred to new culture well without recombinant proteins to avoid confusing them with mother during subsequent observations. The fecundity, offspring and male ratio were counted every day until in all experiments rotifers died. Based on the experimental data, reproductive phase, post- reproductive phase and lifespan were calculated. Duncan's multiple range test using SPSS was conducted to identify significant differences ($p<0.05$) among treatments.

Population culture

Flask cultures were inoculated 50 ml of 15 psu water containing 5 rotifers/ml and incubated at 28°C incubator under darkness without changing the medium. Recombinant MSTN prodomains were 0 (control), 0.5, 1, 2 and 4 μg/ml and Tris-HCl (pH 7.0) buffer was used as a solvent control. In order to confirm the recombinant proteins effects on population growth, rotifers were counted every other day until day 8. All experiments were in triplicates. Duncan's multiple range test using SPSS was conducted to identify significant differences ($p<0.05$) among treatments.

Results and Discussion

In order to investigate the impacts of recombinant MSTN prodomains on rotifer reproduction, recombinant MSTN prodomains of various concentrations were treated on isolated individuals. Rotifer (*B. rotundiformis*) treated with 1, 2, and 4 μg/ml pMALc2x-poMSTNpro was significantly shortened the time of appearance the first egg (pre-reproductive phase) compared to that of non-treated one, but low concentrations 0.25, 0.50, and 0.75 μg/ml of pMALc2x-poMSTNpro had no significant effects. In pMALc2x-poMSTNpro treated rotifer, lifespan was shortened and percentage of male was increased overall in dose-dependently. Especially, pMALc2x-poMSTNpro at 4 μg/ml resulted in a significantly decrease in reproductive-phase, fecundity and offspring. However, there were no significant difference in reproductive phase, post-reproductive phase, offspring, lifespan and fecundity with pMALc2x-poMSTNpro treatments except at 4 μg/ml concentration and control groups (Table 1). Also, pMALc2x-sMSTNpro was significantly effective in pre-reproductive phase from 0.25 μg/ml to 4 μg/ml compared to those of pMALc2x-poMSTNpro treated groups and control ones. And reproductive phase, offspring, lifespan and fecundity of pMALc2x-sMSTNpro treated groups were increased than those of the control ones. The percentage of male in pMALc2x-sMSTNpro treated rotifer groups was increased in dose-dependently like that of pMALc2x-poMSTNpro. But no significant difference in post-reproductive phase (Table 2).

To determine the effects of two recombinant MSTN prodomains on its population growth, flask culture of the rotifer (*B. rotundiformis*) was carried out. pMALc2x-poMSTNpro and pMALc2x-sMSTNpro had a significant effect on the population growth of rotifer *B. rotundiformis*. The increase in population growth by 0.5 μg/ml pMALc2x-poMSTNpro treated (300±8.82 and 446.67±21.43 individuals/ml for day 4 and day 6, respectively) (Fig. 1 and 2) and 1 μg/ml pMALc2x-sMSTNpro treated (297.78±50.04 individual/ml

Table 1. Effects of pMALc2x-poMSTNpro on reproduction and life cycles of Rotifer, *B. rotundiformis*

poMSTMpro concentrations (μg/ml)	Pre-reproductive phase (hr)	Reproductive phase (day)	Post-reproductive phase (day)	Offspring (ind.)	Life pan (day)	Fecundity (♀♀)	Male ratio (%)
Control	27.8±0.57^{d}	6.8± 0.52^{d}	2.5±0.39^{a}	9.2± 1.09^{cde}	10.1± 0.41^{cd}	14.1±1.72^{de}	0.0±0.00^{a}
Tris buffer	24.1±0.67^{c}	6.6± 0.34^{cd}	2.7±0.39^{a}	9.6± 0.65^{de}	10.3± 0.44^{d}	13.5±0.92^{cde}	0.0±0.00^{a}
0.25	24.9±0.43^{c}	5.7± 0.29^{bcd}	2.5±0.27^{a}	6.8± 0.52^{bc}	9.3± 0.31^{bcd}	10.4±0.81^{bcd}	0.0±0.00^{a}
0.50	24.5±0.50^{c}	5.8± 0.42^{bcd}	2.1±0.23^{a}	7.1± 0.86^{bc}	8.8± 0.40^{bc}	10.2±0.32^{bc}	2.0±0.10^{b}
0.75	23.1±0.63^{bc}	5.0± 0.36^{b}	2.3±0.29^{a}	6.3± 0.48^{b}	8.3± 0.32^{b}	8.8±0.85^{b}	8.0±0.35^{c}
1	19.8±0.21^{a}	6.5± 0.35^{cd}	1.9±0.25^{a}	11.0± 0.99^{e}	9.2± 0.39^{bcd}	15.8±1.45^{e}	8.8±0.25^{c}
2	19.7±0.22^{a}	5.4± 0.44^{bc}	2.0±0.19^{a}	8.3± 0.85^{bcd}	8.2± 0.48^{b}	11.6±1.33^{bcd}	9.0±0.50^{c}
4	22.2±1.17^{b}	3.3± 0.43^{a}	2.3±0.40^{a}	3.8± 0.45^{a}	6.5± 0.59^{a}	4.8±0.60^{a}	15.0±0.42^{d}

*Values (mean±S.E. n=24) in the same column not sharing a common superscript are significantly different (p<0.05). Control contained food suspension only, without recombinant protein.

Table 2. Effects of pMALc2x-sMSTNpro on reproduction and life cycles of Rotifer, *B. rotundiformis*

sMSTNpro concentrations (μg/ml)	Pre-reproductive phase (hr)	Reproductive phase (day)	Post-reproductive phase (day)	Offspring (ind.)	Life span (day)	Fecundity (♀♀)	Male ratio (%)
Control	21.9 ±0.69^{e}	4.9 ± 0.51^{a}	2.0 ±0.23^{a}	3.3 ±0.61^{a}	7.6 ± 0.46^{a}	4.4 ±0.87^{a}	0.0 ±0.00^{a}
Tris buffer	19.5 ±0.42^{cd}	5.0 ± 0.51^{a}	1.8 ±0.27^{a}	2.8 ±0.26^{a}	7.6 ± 0.44^{a}	4.0 ±0.40^{a}	0.0 ±0.00^{a}
0.25	19.2 ±0.51^{bcd}	9.4 ± 0.47^{c}	2.0 ±0.23^{a}	13.5 ±1.73^{bc}	12.2 ± 0.51^{c}	20.1 ±2.57^{bc}	1.0 ±0.02^{b}
0.50	17.9 ±0.49^{ab}	9.3 ± 0.46^{c}	1.8 ±0.17^{a}	23.0 ±1.75^{f}	11.9 ± 0.44^{c}	33.6 ±2.50^{e}	1.2 ±0.05^{b}
0.75	18.2 ±0.62^{abc}	8.5 ± 0.51^{bc}	1.6 ±0.16^{a}	17.3 ±1.73^{cd}	10.8 ± 0.56^{bc}	25.3 ±2.61^{cd}	2.0 ±0.25^{c}
1	17.3 ±0.45^{a}	8.4 ± 0.63^{bc}	1.8 ±0.25^{a}	13.7 ±1.01^{bc}	11.0 ± 0.69^{bc}	19.7 ±1.57^{bc}	3.0 ±0.50^{cd}
2	18.1 ±0.46^{abc}	8.3 ± 0.60^{bc}	1.7 ±0.19^{a}	13.3 ±1.22^{b}	10.9 ± 0.57^{bc}	18.9 ±1.80^{b}	3.5 ±0.22^{d}
4	17.7 ±0.45^{ab}	7.5 ± 0.63^{b}	1.6 ±0.16^{a}	10.5 ±0.85^{b}	9.8 ± 0.67^{b}	14.5 ±1.30^{b}	5.5 ±0.35^{f}

*Values (mean±S.E. n=24) in the same column not sharing a common superscript are significantly different (p<0.05). Control contained food suspension only, without recombinant protein.

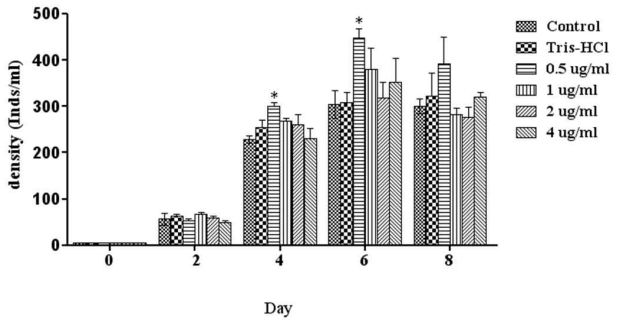

Fig. 1. Population growth of *B. rotundiformis* treated with pMALc2x-poMSTNpro at various concentrations. Level of significant difference (*p<0.05) of each treatment compared with the control on the same day is indicated top of the bars. All experiments were in triplicates.

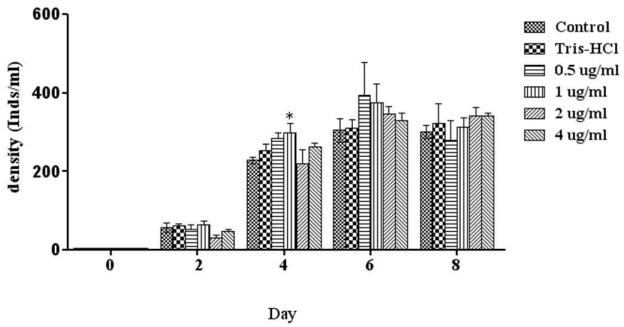

Fig. 2. Population growth of *B .rotundiformis* treated with pMALc2x-sMSTNpro at various concentrations. Level of significant difference (*p<0.05) of each treatment compared with the control on the same day is indicated top of the bars. All experiments were in triplicates.

for day 4) was significantly greater than that by control (227.78±7.78 and 304.44±29.90 individuals/ml, day 4 and day 6, respectively) (Fig. 2).

The switch from asexual to sexual reproduction is triggered by external signals which excreted into the medium by the rotifers themselves as quorum sensing in bacteria [15]. A 39 kD amixis inducing protein (MIP) was isolated that the 17N-terminal amino acids of this protein were strong similarity to a steroidogenesis inducing protein reported from human follicular fluid [28]. Thus MIP likely acts an important role in steroidogenesis, probably by binding to a membrane receptor that triggers a signal transduction. As the rotifer signal transduction molecules were through to be similar to hormones characterized in other animals, this led to an examination of several animal hormones for their effect on the rotifer *B. plicatilis* [6-10]. These authors investigated the effects of vertebrate growth hormone, human chorionic gonadotropin, 17β-estradiol, and triiodothyronine, as well as the insect steroid 20-hydroxyecdysone, 5-hydroxytryptamine (5-HT or serotonin) and gamma aminobutyric acid (GABA) and juvenile hormone on rotifer sexual female production and body size. Juvenile hormone significantly increased the rate of mixis to about 30% from a 10% rate in the control when exposed at 0.05 and 0.5 μg/l, but at concentrations of 50 mg/l mixis rates significantly decreased compared to controls. It was reported that increase of the mixis rate in individual rotifer cultures from 4% in controls to 8% in the F2 generation of females exposed to 5 and 50 mg/l juvenile hormone. They showed that exposure to 50 mg/l 17 β -estradiol increased the mixis rate to about 2-fold compared to controls, but lower concentrations had no effect. GABA at 50 mg/l increased rotifer asexual reproduction in stressful environmental conditions such as low food and high free ammonia levels as in mass cultures. In contrast, 5-HT enhanced sexual reproduction.

In this study, capacity of recombinant MSTN prodomains to impact rotifer reproduction also was examined. Treatment of recombinant MSTN prodomains for rotifer resulted in shortened the time of pre-reproductive phase and also observed reproductive effects in lifespan and percentage of male in dose-dependently compared to those of control one. These results propose that the shorter pre-reproductive phase attribute to promote the reproductive cycle in the initial growth of *B. rotundiformis*, further it will be increase the population numbers. So, the growth promoting activity of recombinant MSNT prodomains on *B. rotundiformis* was measured and the results showed a significantly increase on the population growth of rotifer *B. rotundiformis* by them. Most rotifers have mechano- and chemoreceptors, which are sensitive to environmental stimulations and in direct contact with external medium [4]. Even if the molecular weights of recombinant MSTN prodomains are large, if they have a receptor in *B. rotundiformis*, then would have an effect. These observations of significant biological effects by exposure of recombinant MSTN prodomains on *B. rotundiformis*, suggest the presence of the MSTN signaling cascade in rotifers that regulates reproduction and growth. But presence of MSTN, receptors and molecular mechanisms are yet unknown, therefore to understand the effects of MSTN on *B. rotundiformis*, more studies are needed.

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초록 : Myostatin prodomains이 rotifer 생활사에 미치는 영향

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Myostatin (MSTN)은 TGF (transforming growth factor)-beta family에 속하며, 골격근 성장의 억제 조절인자로서 여러 포유류에서 MSTN 유전자 돌연변이는 골격근 증가를 유도한다. MSTN prodomain은 MSTN의 생물학적 활성을 저해하는데, MSTN prodomain이 과 발현된 쥐에서 과도한 근육축적이 확인되었다. 로티퍼(rotifer; *Brachionus rotundiformis)*는 치어기 어류의 양식산업에 있어 주요한 일차적 먹이생물이다. 그러나 로티퍼에서 MSTN 및 MSTN prodomain의 기능과 발현 유무는 알려져 있지 않다. 따라서 본 연구는 재조합 MSTN prodomains이 로티퍼에 미치는 영향에 관하여 조사하고자 하였다. 로티퍼 개체배양 실험을 통하여 재조합 MSTN prodomains (pMALc2x-poMSTNpro, pAMLc2x-sMSTNpro)에 의한 로티퍼의 생식 전 단계, 순 생식단계, 생식 후 단계, 산란, 수명, 포란, 수컷 발생률을 확인하였으며, 또한 pMALc2x-poMSTNpro와 pAMLc2x-sMSTNpro이 밀집배양에서 로티퍼의 개체성장에 영향을 미치는지에 대하여 확인하였다. 그 결과 농도가 1, 2, 4 μg/ml에서 pMALc2x-poMSTNpro를 처리한 실험군과 0.25 μg/ml에서 4 μg/ml 농도까지 pMALc2x-sMSTNpro를 처리한 실험군에서 로티퍼의 생식 전 단계가 아무처리하지 않은 대조군에 비하여 짧아졌다. 밀집배양 실험에 있어 pMALc2x-poMSTNpro와 pMALc2x-sMSTNpro 모두 로티퍼의 개체 수를 증가를 유도하여, 재조합 MSTN prodomains에 의해서 로티퍼의 reprodution에 영향을 주는 것으로 나타났다. 하지만, 재조합 MSTN prodomains이 어떠한 수용체를 이용하여 신호를 전달하는지에 대한 연구는 앞으로 더 진행되어야 하며, 본 연구의 결과는 재조합 MSTN prodomains이 미세조류에서의 기능 및 메커니즘연구에 중요한 기초자료가 될 것으로 사료된다.