

牙鲆肌肉生长抑制素(MSTN)基因克隆

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摘要: 采用同源克隆及基因组步移的方法, 分离克隆了牙鲆肌肉生长抑制素(MSTN)基因。经过序列分析及 cDNA 验证, 牙鲆 MSTN 基因具有 3 个外显子和 2 个内含子, 编码 377 个氨基酸。5' 侧翼区含有 8 个 TATA 框, 一个 CAAT 框, 6 个 E 框; 3' 侧翼区含有加尾信号。通过同源分析, 牙鲆 MSTN C 末端含有 9 个保守半胱氨酸残基和一个 RVRP 蛋白酶酶切位点; 通过进化树分析, 牙鲆 MSTN 与鱼类 MSTN 基因聚为一支。RT-PCR 分析表明, 牙鲆 MSTN 在胚胎发育中不表达或表达量较低, 说明 MSTN 在牙鲆胚胎发育中并不起重要作用; 其在各组织中的表达, 随个体和环境的不同而有差异, 暗示 MSTN 的表达受外界因素调控。

关键词: 牙鲆; 肌肉生长抑制素; 克隆

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肌肉生长抑制素(myostatin, MSTN)基因, 又称 GDF8, 是转录生长因子(TGF- β)家族的成员之一。自 1997 年在小鼠中发现该基因^[1]至今, 大量的实验证据表明该基因对哺乳动物骨骼肌的发育和生长有负调控作用, 该基因的缺失可导致骨骼肌增生^[2]。在小鼠中, 通过基因敲除使 MSTN 基因 C 端生物活性区失活, 从而使小鼠的骨骼肌比普通野生小鼠增加了 2~3 倍^[1,3]; 在牛和狗中, 该基因的自然突变导致的基因失活, 使其肌肉产量增加^[4-6]; 在斑马鱼(*Brachydanio rerio*)中, MSTN 基因的沉默可以导致肌肉数量和体积的增加^[7-8]。到目前为止, 除了在哺乳类^[9]和鸟类^[10]上有 MSTN 的报道外, 还有大量鱼类 MSTN 基因被克隆: 斑马鱼^[11], 虹鳟(*Oncorhynchus mykiss*)^[12], 大西洋鲑(*Salmo salar*)^[13], 莫桑比克罗非鱼(*Oreochromis mossambicus*)^[14], 白鲈(*Morone americana*)^[15], 条纹石鲈(*Morone saxatilis*)^[15], 金头鲷(*Sparus aurata*)^[16], 鲶(*Ictalurus punctatus*)^[17-18], 波纹

短须石首鱼(*Umbrina cirrosa*, Sciaenidae)^[19], 石斑鱼(*Epinephelus coioides*)^[20], 鲈(*Lateolabrax japonicus*)^[21]等。通过比对分析发现, MSTN 基因的蛋白酶酶切位点(RXXR)和 C 末端的半胱氨酸残基在不同的物种中都保持着高度保守^[4]。

与其它的 TGF- β 家族的成员蛋白一样, MSTN 以前体形式合成后被蛋白酶酶切, 形成 N 端前肽和 C 端活性成熟肽^[1,22-23]。酶切之后, 前肽与成熟肽仍以非共价结合的方式结合。MSTN 前肽对于 MSTN 形成正确的二聚体极其重要^[24]; MSTN 前肽还可以通过与成熟肽结合, 从而抑制 MSTN 与其受体结合, 阻断其功能^[25]。通过转基因表明, MSTN 前肽可以抑制斑马鱼肌肉生长^[11]。

在鱼类中, MSTN mRNA 可以在肌肉、眼、脑、肠、鳃、肾、心和脾中发现。尽管不同物种的表达谱存在差异, 但在肌肉中都检测到了较高的表达^[12-14,16,19,21]。并且在斑马鱼^[11,26]、鲈^[12-13], 金头鲷^[16], 波纹短须石首鱼^[19]中都发现了两种类

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型的 MSTN。MSTN 广泛的分布及其不同类型的存在表明,鱼类中 MSTN 除了参加肌肉的生长和发育调控外,还可能参与机体的其它功能。但到目前为止,鱼类 MSTN 的功能仍没有完全弄清楚。由于 MSTN 对生物体肌肉生长的调控作用,人们期望通过对该基因的控制来增加肌肉产量。鉴于 MSTN 在水产养殖中潜在的应用前景,实验克隆了牙鲆 MSTN,并对其进行了初步分析。

1 材料与方法

1.1 引物设计

根据不同鱼类 MSTN 的保守区,设计了两对引物,用于牙鲆 MSTN 同源克隆。在获得序列的基础上,再设计基因特异引物,用于基因组步移和全长开放阅读框的扩增。引物序列及其用途如表 1。

表 1 牙鲆 MSTN 基因克隆及分析引物
Tab. 1 Primers for MSTN clone and analysis in Japanese flounder

引物名称 primer	引物序列 primer sequence	退火温度(°C) T _m	用途 purpose	片断长度(bp) fragment length
MSTN2-5'	5'-CTGCAAATCTCCCGCCTGATGCC-3'	56	同源克隆外显子 2 和内含子 2	1334
MSTN2-3'	5'-TCTTGCCGTAGATGATCTGCTC-3'			
MSTN3-5'	5'-TGAAGACTTTGGCTGGGACTGGATC-3'	57	同源克隆外显子 3 和 3'UTR	771
MSTN3-3'	5'-CTTGAGGATTCTGGTTTCACT-3'			
MSTN3W	5'-TTGAGGACTTTGGCTGGGACTGGATT-3'	67	牙鲆 MSTN 3'端 基因组步移	1414
MSTN3WN	5'-AATCGCAAAGAGCAGATCATCTACGGC-3'			
MSTN5W1	5'-CCCAGCCAAAGTCTCAAAGTCAACC-3'	67	MSTN 5'基因组 第一次步移	1112
MSTN5WN1	5'-CCGGCGTTCACGTCAATCTTCAGGGA-3'			
MSTN5W2	5'-GGCTGCTGATGGTGGCTCTCTTGGTC-3'-3'	67	MSTN 5'基因组 第二次步移	929
MSTN5WN2	5'-CAAACCATAAAGGGACGCAGACTGTGAGC-3'			
MORF5	5'-ATCAAACCTCCACAGAGAAAATG-3'	58	MSTN ORF 扩增 及 RT 分析	1474
MORF3	5'-CCGCTACTCTGGTTCTATTGCAC-3'			
Jactin-F	5'-TGGCATCACACCTTCTAC-3'	60	牙鲆 RT 分析 actin 内参	429
Jactin-R	5'-CTGCATCTCTGCTCAAAGTC-3'			

1.2 基因组 DNA 提取及各组织 cDNA 制备

取 2 龄牙鲆活体各组织,液氮速冻后 -80 °C 保存。利用高盐法提取牙鲆肝脏 DNA:液氮研磨肝脏组织成粉末后,放入 1.5 mL 离心管中。然后向每管中加入 0.9 mL 裂解液(10 mmol·L⁻¹ Tris-HCl, 400 mmol·L⁻¹ NaCl and 2 mmol·L⁻¹ Na₂EDTA, pH 8.2), 0.1 mL 10% SDS 和 100 μg 蛋白酶 K, 55 °C 水浴过夜。消化完全后,向每管中加入 270 μL 饱和 NaCl(6 mol·L⁻¹),混匀, 12 000 r·min⁻¹ 离心 30 min。将上清转移到一新的离心管中,加入两倍体积无水乙醇沉淀 DNA,

75%乙醇洗涤一遍,溶于 TE 备用。

选取牙鲆胚胎发育中的囊胚期、原肠期、尾芽初期、尾芽末期、心跳期和出膜期胚胎,保存于 -80 °C 备用。选取 3 月龄饥饿 1 个月牙鲆个体、6 月龄正常饲养牙鲆个体和 2 龄饥饿 1 周牙鲆个体的性腺、脑、肌肉、心脏、肠、肾、肝、脾、眼、鳃、血液等组织,保存于 -80 °C 备用。利用 Trizol (Invitrogen)法提取牙鲆各组织 RNA,具体操作步骤按照 Trizol 说明书进行。用 MMLV (promega)反转录酶的随机引物法合成 cDNA 第一链,具体操作步骤按照说明书进行。

1.3 克隆及测序

PCR 按照普通程序进行,退火温度按照表 1 中进行,延伸时间按照预期片段设定(1 kb 需 1 min)。基因组步移依照 Genomwalker Universal Kit(Clontech)说明书进行。所有 PCR 反应均在 PTC-200 型 PCR 仪上进行。

PCR 产物经 1% 琼脂糖凝胶电泳纯化后,用 QIAEX II Gel Extraction Kit (QIAGEN) 凝胶回收试剂盒回收。将回收的产物按照摩尔比 3:1 的比例与 PBS-T(天为时代)载体连接,并将连接产物转化感受态细胞 DH5 α 。利用载体测序引物进行菌液 PCR,筛选阳性单克隆送上海联合基因公司测序。

1.4 序列分析

利用 DNAMAN 软件去除载体序列,按照序列比对和 GT-AG 内含子外显子规则剪除内含子。利用 MEGA 3.1 构建进化树。各基因 Genbank 序列号为:斑马鱼 GDF11 (NM_212975),恒河猴 GDF11(XM_001096135),挪威鼠 GDF11 (XM_343148),家鼠 GDF11(MMGDF11S03),狗 GDF11(XM_843265),沟鲈 MSTN(AF396747),鸡 MSTN(AY448007),银鲑 MSTN-1(AY434465),银鲑 MSTN-2 (AF394687),河豚 MSTN-1(AY445322),河豚 MSTN-2 (AY445321),家鼠 MSTN(NM_010834),人 MSTN(AF019627),莫桑比克罗非鱼 MSTN (AF197193),虹鳟 MSTN-1(AF273035),虹鳟 MSTN-2(AF273036),黄鲷 MSTN(AAX82170),带纹白鲈 MSTN(AF290910),斑马鱼 MSTN-1(AY258034),斑马鱼 MSTN-2(AY687474)。

2 结果

2.1 牙鲈 MSTN 基因克隆及特征

通过两对 MSTN 保守区引物的扩增及基因组步移,获得了牙鲈 MSTN 全长基因组 DNA 序列,并通过基因特异引物在牙鲈 cDNA 中验证了开放阅读框的正确性。最终获得了 4 932 bp 的基因组序列,其中包括 1 340 bp 5'侧翼序列,382 bp 的外显子 1,361 bp 内含子 1,371 bp 外显子 2,757 bp 内含子 2,381 bp 外显子 3,1 340 bp 3'侧翼序列。开放阅读框共长 1 134 bp,编码 377 个氨基酸(图 1)。5'侧翼区含有 8 个 TATA 框,一个 CAAT 框,6 个 E 框 (MyoD 结合位点, CAXXTG)。在很多物种中发现的位于加尾信号

和多聚 A 间的保守序列 (YGTGTTY),也在 MSTN 3'端存在^[27]。

2.2 牙鲈 MSTN 蛋白比对及进化树构建

经进化树分析(图 2),牙鲈 MSTN 与鱼类 MSTN 紧密聚为一支,而与其高度同源物 GDF11 同源性较远,说明我们克隆得到的基因属于 MSTN 基因。通过 Clustal X 蛋白序列比对发现(图 3),牙鲈 MSTN 拥有 9 个保守半胱氨酸残基和 RXXR 蛋白酶切位点,并具有保守的 C 末端。牙鲈 MSTN 与其它物种的同源性为:人 64.5%,家鼠 63.5%,鸡 64.5%,斑马鱼 II 型 65.5%,斑马鱼 I 型 80.1%,虹鳟 II 型 83.5%,虹鳟 I 型 84.5%,白鲈 92.6%,黄鲷 90.5%,沟鲈 77.5%。通过同源性数据,发现牙鲈 MSTN 基因与斑马鱼和虹鳟的 I 型 MSTN 较为接近,说明我们克隆的牙鲈 MSTN 应该属于 I 型 MSTN。

2.3 牙鲈 MSTN RT-PCR 分析

对牙鲈不同发育时期,及不同个体的不同组织进行了 RT-PCR 分析,PCR 产物经测序证明无非特异产物或交叉扩增。用于扩增内参的 actin 引物,在设计时跨越了一个内含子序列,所以在表达时可以用来检测是否有 DNA 污染(图 4-A)。在胚胎发育中,没有检测到 MSTN 的表达(图 4-B)。在 3 月龄长期饥饿个体(不投饵饲养 1 月,其余条件与正常相同)组织中(图 4-C),在脑和肾中的表达最强,在肌肉和眼中的表达次之,在脾中没有检测到表达。在 6 月龄正常饱食鱼体(正常饲养)组织中(图 4-D),在肾和眼中检测到了较强的表达,在肌肉中的表达次之,在脑、肾、脾和鳃中都检测到了非剪切转录本,在心脏和肝中没有检测到转录本。非剪切状态的转录本经过测序得到证实。在 2 龄短期饥饿鱼(不投饵饲养 1 周,其余条件与正常相同)组织中(图 4-E),在脑中检测到了较强的表达,而在肾和肌肉中仅检测到了微弱的表达。

3 讨论

克隆分离得到的牙鲈 MSTN 基因与其它物种的 MSTN 具有较高的同源性,特别是其 C 端活性功能区。牙鲈 MSTN 拥有 3 个外显子和 2 个内含子,编码 377 个氨基酸。推定的蛋白质拥有 RXXR 蛋白酶切位点和 9 个保守半胱氨酸残基,与其它物种的 MSTN 特征相同^[4,28-29]。其 5'侧

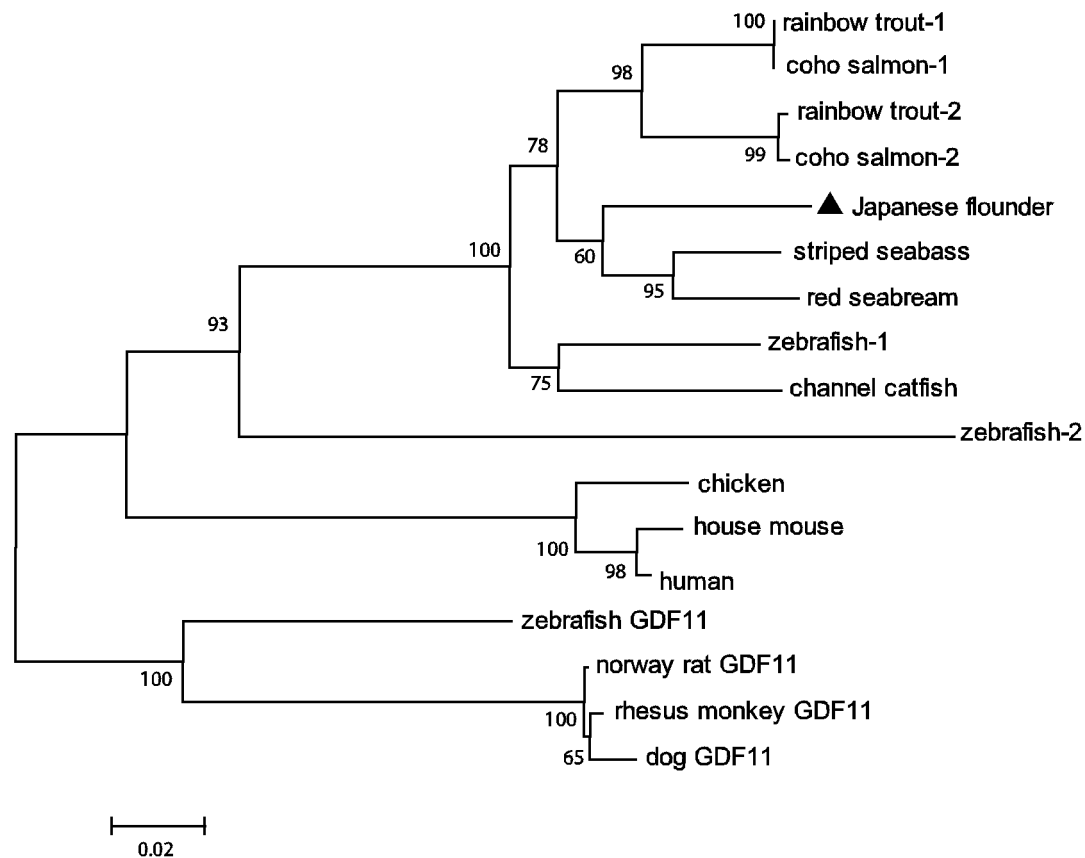


图 2 牙鲆 MSTN 进化树分析

Fig. 2 Phylogeny tree of Japanese flounder MSTN

翼区拥有 8 个 TATA 框、一个 CAAT 框和 6 个 E 框,类似于在斑马鱼和溪红点鲑 (*Salvelinus fontinalis*) 上的报道^[11,30];其 3' 端含有加尾信号。通过同源性和进化树分析,牙鲆 MSTN 基因与鱼类 MSTN 基因有较高的同源性,说明了该基因属于 MSTN 基因并且应该归属于 I 型 MSTN。通过序列分析,发现来自脑和肌肉的 MSTN 基因具有两个 SNP 位点 (Genbank Accession No. EF668005, EF668006),一个发生在编码区,为同义突变;另一个发生在 3' 非编码区。

用于 MSTN 表达的引物,跨越整个编码区,而且还可以通过 PCR 产物片段的大小区分转录本的加工状态。如果表达产物与预期 cDNA 长度相符,则说明转录本内含子均被剪切;而如果表达产物与预期 DNA 长度相符,则说明转录本处于未剪接状态,内参 actin 可以检测是否有 DNA 污染。在胚胎发育中,没有检测到 MSTN 的表达 (图 4-B),说明 MSTN 在牙鲆胚胎发育过程中并不起重要作用,类似的结果也在虹鳟中发现^[31]。

MSTN 在牙鲆脑中有大量表达 (图 4-C, D, E),说明 MSTN 在鱼类神经发生中起着重要作用^[31]。其在肾脏中的高表达 (图 4-C, D),暗示着其在免疫方面的潜在功能^[32]。在肌肉中虽然检测到了表达,但其表达量较低 (图 4-C, D, E)。有研究表明,长期禁食可降低幼鱼 MSTN 的表达,短期禁食可增加幼鱼 MSTN 的表达,而长期禁食对成鱼却没有影响^[33]。实验选择了不同时期,不同状态的鱼体进行了表达分析 (正常饱食,短期饥饿 1 周,长期饥饿 1 月),发现不同状态下,其表达图谱和表达强度各有不同 (图 4-C, D, E),说明 MSTN 的表达受外界营养状况的影响。长期饥饿 3 月龄幼鱼的成长受到明显阻碍,其肌肉 MSTN 表达水平较正常有降低的趋势 (图 4-C 和图 4-D 中肌肉 MSTN 表达量与 actin 表达量对比);而短期饥饿的 2 龄成鱼也有下降的趋势 (图 4-D 和图 4-E 中肌肉 MSTN 表达量与 actin 表达量对比)。在正常养殖的个体中,发现存在非剪接的转录本 (剪接长度约 1 270 bp,非剪接长度约 2.4 kb) (图 4-D)。处于非剪接状态的转录本经测序证明其

Japanese flounder	—MHLSHIVLYL SLLVALGPVVLSDQETHHQ—	QPSASSPEDAE	QCATCDVRQQIKTMRNAIKSQILSKLRMKEAPNISRDIVKQLLPK	86
channel catfish	—...AQVLIS.GFV..F..MART.TGAPE.QQ—	Q.QPTAVT.ER.AQCSAASA.AF..HS.QL..Q.....L.Q...V...V.....		93
mozambique tilapia	—...Q.....I.....A.Q.....	V.T.V.TD.....E.....E.....		85
red seabream	—...P.Q.....IV.....QQ.QQQQQQ.....TG...T.....E.....			93
striped seabass	—...Q.A.....I.....Q.....T.....T.....E.....			85
rainbow trout-1	—...TQVLI..FM..F..G.G..TA.....	P.TD—G.....S.E.....N..H.....L.Q.....V.....		83
rainbow trout-2	—...N.MQVLI..FM..F..MG.G..TA.....	SP.TD—G.....S.E.....N..H.....L.H.....V.....		83
zebrafish-1	—...FTQVLIS..V.I.C..GYG.ITA—	H.QPSTAT.ES.—L.S.EF..HS.L..H.....L.Q.....V.....		84
zebrafish-2	—...F.LFYLSFWGV.GSQNQNLSTTTT.TT—	AFVTPGD.NG—T..QF..S.LL..HS.....I..LEQ.....T..L...		85
fugu rubripes	—...Q..PSM.HF..MIS.SL...G...Q.....	PVG...T.....V.....H.....P.....T.....		85
fugu rubripes-2	—...M.V.AV.TVVSAGFSMEMNQTS—	RLL.E.G.—SA..F.EHS.Q..HS.....I..LEQ.....MIR....		76
chicken	—MQKLAVVYI..FMQI.VD..A.DGSSQPT—	ENAEKDG—L.NA.TW..NT.SS.IE..I.....LEQ.....VI.....		83
house mouse	—MQKLQMYVYI..FM.I.A...D.NEGSERE—	ENVEKEG—L.NA.AW..NTRYV.IE..I.....LET.....K.AIR...R		84
human	—MQKLQCVYI..FM.IVA...D.NENSEBQK—	ENVEKEG—L.NA.TW..NT.SS.IE..I.....LET.....K.VIR....		83
Japanese flounder	APPLQQLLDQYDVLGDDNRDY—	VMEDDDEHATTETIMMATEPEPIVQVDAEPKCCFFSFTQKFKASRIVRAQLWVYL.RPADEATTVFLLQISRLM—		180
channel catfish	...V...L.....GKPGTALQDEE..E.....V.S..A.N.D...QK.....	SP.I.....H.....		192
mozambique tilapia	...EE—L.....V.V...D.A...GQ.....I.....V.....H..SE.V.....I—			179
red seabream	...E...I.....SV...G..R.....I..N.....H..AS.....—			187
striped seabass	...I.....S...G..R.....N.....H..QS.....—			179
rainbow trout-1	...K.G—L..E...I...T...S...R.K...L..SS.I.VN..H...H.L..V.....—			177
rainbow trout-2	...K.....K.G—L..E...I...T...S...GK.....NS.I..N...H.Q.P..V.....I—			177
zebrafish-1	...SK.G—AV.E...T...D...RK.....SP.I..N...H..E.....—			178
zebrafish-2	...E...QN.—G—I-SE.E.Q.SS..IT...QA.T.LVGM...M.ALSP..ILPDS.LK.L..I...E.P..YI..H.E—			173
fugu rubripes	...T.E...I.....ASV...NG.....V..L.....H..A.....—			179
fugu rubripes-2	...T.....PR—VE.ED...IT...K.N..A.DA-LSS..LL.LSP.I.PKN.L.L..H...TVA.....K—			160
chicken	...E.I...QR..SS.G—SL...Y...IT.P..SDFL..MEGK...K.SS.I.YNKV.K...I...QVQKP...V..L..IK			178
house mouse	...RE.I...QR..SS.G—SL...Y...IT.P..SDFLM.A.GK...K.SS.I.YNKV.K...I...VKTP...V..L..IK			179
human	...RE.I...QR..SS.G—SL...Y...IT.P..SDFLM..GK...K.SS.I.YNKV.K...I...VETP...V..L..IK			178
Japanese flounder	PVTDGSR-HIRIRSLKIDVN-AGLSSWQSIDVKQVLTWVLRQPETNWCIEINAFDSRGNDLAVTSTEPGEEGLQPFMEVKITDGP	KRVRRDAGLDCDENS		278
channel catfish	.IK..R.-V.....D..V.....A.....KS...I..A.....L..L...SEV...T..ES.....			290
mozambique tilapia	...N...S.....-P.PA.....A.....SE..R.A..S.....			277
red seabream	...NG..H.....-V.G.....S.....Q.....A..D.....SE...S.....			285
striped seabass	...N...EL...V.....S.....A.....SE..R.A..S.....			277
rainbow trout-1	...G...G.....-V.....S.....K.....A.A...T..SE...F...S.....			274
rainbow trout-2	...G..-N.Q.....-V.....S.....D.....L..K.....A.A...SE...S...S.....			274
zebrafish-1	...K..G...-VT.....K...R...Y.AK.....T..D..L...SE...I..S.....			275
zebrafish-2	SSSE.NN-S..AQ...-RTN..H..M..L.KL..K..QS.F...K..AN.....S.....L...S.TG..S..T...H			271
fugu rubripes	...N...L..K...V.....S.....K.....Q.....SE..R...L.....			277
fugu rubripes-2	.GIE.NNTRV.V...R..TDT..AG...V.I.SL.QA.....Y.....K.E.R...L.....I...LNS...S..ES..N..E			260
chicken	.MK..T.-YTG...L.M.-P.TGI...T..QN..K..S.L...K..ET.R...FPG...D..N..L..RV..T..S..F...H			276
house mouse	.MK..T.-YTG...L.MS-P.TGI...T..QN..K..S.L...K.L.EN.H...FPG...D..N..L..V..T..S..F...H			277
human	.MK..T.-YTG...L.M.-P.TGI...T..QN..K..S.L...K.L.EN.H...FPG...D..N..L..V..T..S..F...H			276
Japanese flounder	PETRCRCRYPLTVDFEDFGWDWI IAPKRYKANYCSGCEYMHQLQKYPHTLVNKANPRGTAGPCCPTKMSPINMLYFNKKEQIIYGKIPSMVMDRCGCS			378
channel catfish	S.S.....D.V.....G.....			390
mozambique tilapia	..S.....			377
red seabream	..S.....			385
striped seabass	..S.....			377
rainbow trout-1	..S.....			374
rainbow trout-2	..S.....			374
zebrafish-1	S.S.....D..Y.....S.....G.....			375
zebrafish-2	T.S.....V—...S.I.....S.....DR.....L...			367
fugu rubripes	..S.....QEQ.....L			377
fugu rubripes-2	A.....E.....R.....FL.P.Q..A..Q.....R.....H...			360
chicken	T.S.....A.....FVF.....HQ.....S.....G.....A.....			376
house mouse	T.S.....A.....FVF.....HQ.....S.....G.....A.....			377
human	T.S.....A.....FVF.....HQ.....S.....G.....A.....			376

图 3 蛋白序列比对图

RXXR 蛋白酶酶切位点用下划线表示,保守半胱氨酸残基用星号表示

Fig. 3 Alignment of protein sequences

RXXR proteolytic cleavage site was underlined, conserved cysteine was indicated with stars

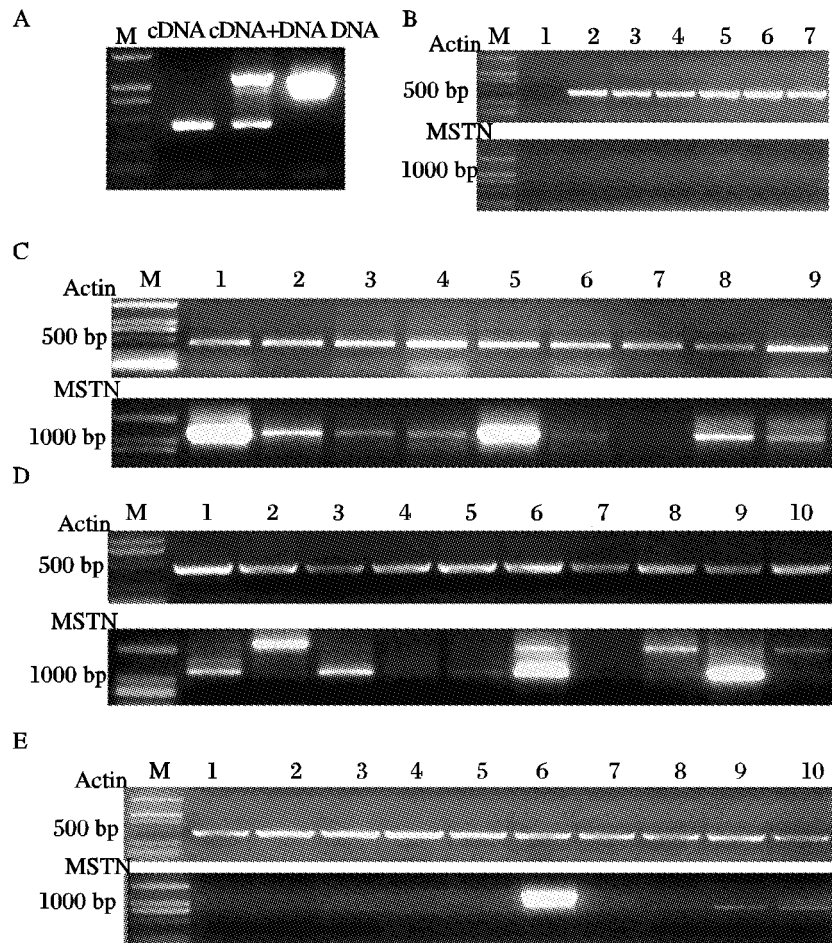


图4 牙鲆 MSTN RT-PCR 分析

A. 牙鲆 actin 内参在牙鲆 DNA 和 cDNA 中的扩增。cDNA: actin 引物在牙鲆 cDNA 中的扩增产物; cDNA + DNA: actin 引物在牙鲆 cDNA 与 DNA 混合物中的扩增产物; DNA: actin 引物在牙鲆 DNA 中的扩增产物。退火温度为 60 度, 30 个循环

B. 牙鲆 MSTN 在不同胚胎发育时期表达分析。M: Marker; 1: 空白对照; 2: 囊胚期; 3: 原肠期; 4: 尾芽初期; 5: 尾芽末期; 6: 心跳期; 7: 出膜期。上侧为 actin 内参扩增产物, 预期长度 480 bp 左右, 30 个循环; 下侧为 MSTN 在不同胚胎发育时期扩增产物, 预期长度 1 270 bp, 60 个循环

C. 牙鲆 MSTN 在 3 月龄, 长期饥饿(1 月) 鱼体内组织表达分析。M: Marker; 1: 脑; 2: 肌肉; 3: 心脏; 4: 肠; 5: 肾; 6: 肝; 7: 脾; 8: 眼; 9: 鳃。上侧为 actin 内参扩增产物, 预期长度 480 bp 左右, 30 个循环; 下侧为 MSTN 在不同组织内的扩增产物, 预期长度 1 270 bp, 60 个循环

D. 牙鲆 MSTN 在 6 月龄, 正常饱食鱼体内组织表达分析。M: Marker; 1: 性腺; 2: 脑; 3: 肌肉; 4: 心脏; 5: 肠; 6: 肾; 7: 肝; 8: 脾; 9: 眼; 10: 鳃。上侧为 actin 内参扩增产物, 预期长度 480 bp 左右, 30 个循环; 下侧为 MSTN 在不同组织内的扩增产物, 预期长度 1 270 bp, 60 个循环; 非转录本预期长度 2.4 kb

E. 牙鲆 MSTN 在 2 龄, 短期饥饿(1 周) 鱼体内组织表达分析。M: Marker; 1: 性腺; 2: 血液; 3: 鳃; 4: 皮肤; 5: 心; 6: 脑; 7: 头肾; 8: 肠; 9: 肾; 10: 肌肉。上侧为 actin 内参扩增产物, 预期长度 480 bp 左右, 30 个循环; 下侧为 MSTN 在不同组织内的扩增产物, 预期长度 1 270 bp, 60 个循环

Fig. 4 RT-PCR analysis of MSTN in Japanese flounder

A. Amplification product of actin in DNA and cDNA of Japanese flounder. cDNA: Amplification product of actin in cDNA; cDNA + DNA: Amplification product of actin in DNA and cDNA; DNA: Amplification product of actin in DNA. $T_M = 60\text{ }^\circ\text{C}$, 60 cycles

B. RT-PCR analysis of MSTN during embryo development. M: Marker; 1: control; 2: blastula stage; 3: gastrula stage; 4: bud early stage; 5: bud late stage; 6: heart beating stage; 7: hatching stage. Predicted actin PCR product upside was 480 bp with 30 cycles; predicted MSTN PCR product downside was 1 270 bp with 60 cycles

C. RT-PCR analysis of MSTN in tissues of 3 months old Japanese flounder with long term starvation(one month). M: Marker; 1: brain; 2: muscle; 3: heart; 4: intestine; 5: kidney; 6: liver; 7: spleen; 8: eye; 9: gill. Predicted actin PCR product upside was 480 bp with 30 cycles; predicted MSTN PCR product downside was 1 270 bp with 60 cycles

D. RT-PCR analysis of MSTN in tissues of 6 months old Japanese flounder with normal feeding. M: Marker; 1: gongd; 2: brain; 3: muscle; 4: heart; 5: intestine; 6: kidney; 7: liver; 8: spleen; 9: eye; 10: gill. Predicted actin PCR product upside was 480 bp with 30 cycles; predicted MSTN PCR product downside was 1 270 bp with 60 cycles and unspliced transcription was predicted as 2.4 kb

E. RT-PCR analysis of MSTN in tissues of 2 year old Japanese flounder with short term starvation(one week). M: Marker; 1: gongd; 2: blood; 3: gill; 4: skin; 5: heart; 6: brain; 7: headkidney; 8: intestine; 9: kidney; 10: muscle. Predicted actin PCR product upside was 480 bp with 30 cycles; predicted MSTN PCR product downside was 1 270 bp with 60 cycles

与 DNA 序列完全相同,包含有各自的内含子,而 actin 内参排除了 DNA 污染的存在,说明该基因在转录后并没有立即进行内含子和外显子的剪接,而是存在非剪接状态。尤其是在脑中,所有转录本均处于非剪切状态,说明 MSTN 转录后调控受到外界因素的影响,从而也暗示了 MSTN 处于多级调控中。牙鲆 MSTN 在胚胎发育中的表达模式更加接近于斑马鱼 MSTN1^[11]。在斑马鱼中,MSTN1 在早期胚胎发育中表达量极低,而 MSTN2 却贯穿表达于整个胚胎发育过程^[11,26]。斑马鱼 MSTN2 的启动子区域拥有 MSTN1 所没有的顺式调控元件(SP1 和 Pit-1a),这些元件可以在肌肉发育中具有活性^[11,26]。因此 MSTN2 可能与骨骼肌的发育更加紧密^[34]。我们克隆到的 MSTN,在表达谱、启动子分析及同源性上更加接近于斑马鱼 MSTN1。其在脑中的高表达,而在肌肉中的相对低表达,也说明其在肌肉发育中可能并不起重要作用。在牙鲆中极有可能也存在第二种 MSTN 类型,其存在性和功能还有待进一步研究。

尽管牙鲆 MSTN 与哺乳类同源性较高,但其表达类型却不完全相同。在小鼠中,MSTN 在骨骼肌中强烈表达,在心肌、乳腺和脂肪组织中有微弱表达^[1]。而在鱼类中,其表达更为广泛,在肌肉、眼、脑、性腺等组织中都有表达^[14,17,30]。所以在鱼类中,MSTN 可能不单单参与了肌肉的调控作用,还参与了其他的机体功能。GDF11 是 MSTN 的同源物,在哺乳类中有着较广的表达谱。通过斑马鱼中 GDF11 的分析,认为其与 MSTN 是起源于同一基因^[26]。在鱼类中,MSTN 可能除了调控肌肉发育外,同时也保留了部分 GDF11 的功能。

MSTN 对于肌肉生长的负调控作用,在哺乳类^[2]和鱼类中都经过了证实^[11]。因此,通过人工控制 MSTN 的活性来增加肌肉产量成为畜牧业和水产业中一大课题。在小鼠中,MSTN 抗体、前肽对 MSTN 的阻断作用明显,并提高了肌肉量^[35],在鱼类中也有类似报道^[11]。牙鲆作为我国重要的养殖鱼类,MSTN 基因的克隆为将来牙鲆肌肉产量控制提供了基础资料。我们克隆到的 MSTN 基因,虽然没有检测到其在肌肉中的明显高表达,但这并不能完全排除其在肌肉生长控制中的作用,牙鲆 MSTN 基因的生物活性还有待于

进一步研究和证实。

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Cloning and expression analysis of the myostatin (MSTN) gene in *Paralichthys olivaceus*

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Abstract: Myostatin (MSTN) gene was isolated from Japanese flounder (*Paralichthys olivaceus*) by homology cloning strategy and genome walking. Two pairs of degenerate primers were designed according to the conserved region of MSTN genes from different fish. Full length DNA sequence of MSTN gene was obtained after one step of 3' genome walking and two steps of 5' genome walking. By sequence analysis and verified in cDNA level, Japanese flounder MSTN gene included three exons and two introns, encoding a 374-amino-acid protein. Eight TATA boxes, one CAAT box and six E boxes were located in 5' flanking region. A polyA signal existed in 3' flanking region. Two single nucleotide polymorphism (SNP) sites were discovered within MSTN cDNA, one located in coding region with synonymous mutation and the other located in 3' untranslated region. Nine conserved cysteine residues and an RXXR proteolytic cleavage domain were detected in the C terminal of protein. MSTN in Japanese flounder was highly homologous with MSTN genes in fish via phylogeny analysis, which had 64.5%, 63.5%, 64.5%, 65.5%, 80.1%, 83.5%, 84.5%, 92.6%, 90.5%, 77.5% identity with human, house mouse, chicken, zebrafish-2, zebrafish-1, rainbow trout-2, rainbow trout-1, striped bass, red seabream and channel catfish, respectively. The MSTN gene of Japanese flounder might belong to type I MSTN, according to identity analysis. The RT-PCR analysis demonstrated that MSTN was undetectable during embryo development, indicating low expression or no expression during embryo development. MSTN had high expression in brain of Japanese flounder, indicating the important role in neural development. The expression of MSTN in kidney indicated the potential role in immunity. However, the expression of MSTN of Japanese flounder in muscle was low. Starvation could induce reduction of MSTN expression in muscle. Unspliced transcript was discovered in normal individual, which was verified by sequencing. The spliced transcript was about 1 270 bp and the unspliced transcript was about 2.4 kb which had all introns. The transcription of myostatin was influenced by environment factors in adult, indicating the transcription was under regulation.

Key words: Japanese flounder(*Paralichthys olivaceus*); myostatin(MSTN); cloning