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Rapid Communication

Transcriptional regulation of skeletal muscle-specific insulin receptor substrate-1 transcript

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1 Introduction

One of the greatest surprises of high throughput transcriptome analyses is that the mammalian genome is pervasively transcribed into many different complex families of RNA. For instance, in protein-coding gene region, there is a large number of alternative transcriptional start sites, resulting in production of not only protein-coding mRNAs but also long non-protein-coding RNAs (lncRNA). In addition to lncRNAs, recent reports reveal a non-coding function for mRNAs; the untranslated region (UTR) of several mRNA transcripts can also function as a trans-acting regulatory RNA, which is independent of their protein-coding functions[1-3]. For instance, we previously reported that the 5'UTR of Vascular endothelial growth factor (VEGF) mRNA silences Stat1 mRNA expression[1].

Muscle differentiation is a powerful system for investigations of lncRNA function and a non-coding function of mRNAs. There is increasing evidence that several lncRNAs and the 3'UTR of mRNA regulate myogenesis through modulation of gene expression required for differentiation, including MyoD[3]. For instance, the 3'UTR of mutant DMPK (dystrophia myotonica protein kinase) mRNA reduces MyoD expression levels, leading to disruption of C2C12 myogenic differentiation[4]. In addition, we previously demonstrated that a transcriptional variant of insulin receptor substrate 1 (*Irs-1*) acts as a regulatory RNA that silences Rb (retinoblastoma) mRNA, resulting in induction of myogenic dedifferentiation[5].

In the present study, we characterized expression profiles of the two different *Irs-1* mRNA (FL-*Irs-1* mRNA, Δ5'-*Irs-1* mRNA) in several mouse tissues and mouse skeletal muscle cell line C2C12. We further elucidated the promoter region of the FL-*Irs-1* transcript.

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2 Materials and Methods

2.1 Cell culture

C2C12 myoblast cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and Penicillin-Streptomycin mixed solution (Nacalai Tesque, Kyoto, JAPAN) at 37℃ with 5% CO_2. At a confluence of 100%, C2C12 myoblast cells were fused by shifting medium to DMEM containing 2% horse serum (differentiation medium) for 3days.

2.2 Plasmid constructs

Promoter activity of *Irs-1* gene was analyzed using a low background promoter less Luciferases plasmid pGL3-basic. A 3.6 kb fragment of the promoter (nt-824 to +2870) was isolated from the mouse *Irs-1* promoter (MG2F1)[6]. p*Irs-1* (-824) promoter, p*Irs-1* (-583) promoter and p*Irs-1* (-25) promoter were prepared by inserting double-strand oligonucleotide *Irs-1* prom (-824) Sac1-S, p*Irs-1* prom (-583) Sac1-S, *Irs-1* prom (-25) Sac1-S and *Irs-1* (-1) -Hind3-AS (Table 1). The PCR products digested with HindIII and SacI restriction enzymes were subcloned upstream of the firefly luciferase gene of pGL3-basic vector (Promega, Madison, WI) (pGL3-*Irs-1* (-824) promoter, pGL3-*Irs-1* (-583) promoter, pGL3-*Irs-1* (-25) promoter).

2.3 Quantitative RT-PCR

Total RNA was extracted from C2C12 cells and male C57BL/6 mice (11-weeks-old) from Japan SLC (Shizuoka, Japan) isolates tissues (adipose tissue, liver, kidney, heart, skeletal muscle; gastrocnemius, tibialis anterio, extensor digitorum longus) with an acid guanidium thiocyanate-

phenol-chloroform mixture (ISOGEN; Nippongene, Tokyo, Japan). Quantitative RT-PCR was performed with the primer and SYBR™ Green dye by using ABI 7300 and 7900 real-time PCR system (Applied Biosystems, Foster City, CA), as described previously[5]. Standards were run in the same plate and relative standard curve method was used to calculate the relative mRNA expression. The list of gene-specific primer sets is shown in Table 1. The Ethics Review Committee for Animal Experimentation of Tokushima University approved the experimental protocols described in this study.

2.4 Transfection

C2C12 cells were transiently transfected with pGL3-*Irs-1* (-824) promoter, pGL3-*Irs-1* (-583) promoter or pGL3-*Irs-1* (-25) promoter using JetPRIME™ transfection regents according to the manufacturer's instructions (Polyplus, Berkeley, CA). Cells were allowed to incubate for 24 hours to experiments.

2.5 Statistical analysis

All data are expressed as the mean ± standard deviation of 3 - 6 individual samples per group. Differences between groups were analyzed via one-way analysis of variance with SPSS (release 6.1, SPSS Japan, Tokyo, Japan). Differences between two groups were tested with Scheffé's test. $P < 0.05$ was considered statistically significant.

Table 1 Primer sets used in this study

Primer name	Sequence
Irs-1 prom (-824) SacI-S	5'-AAAAGAGCTCGCTAATAGTGCCAGGTGTGAGATCCCAG -3'
Irs-1 prom (-583) SacI-S	5'-AAAAGAGCTCTACCTGTTGGGTGAGAGCTAGCAGGTC-3'
Irs-1 prom (-25) SacI-S	5'-AAAAGAGCTCGTTGGGGCTCTCGGCAACTCTCCGA-3'
Irs-1 (-1)-Hind3-AS	5'-AAAAAAGCTTTCGGAGAGTTGCCGAGAGCCCCAACCA-3'
18S ribosomal RNA-S	5'- GCAATTATTCCCCATGAACG -3'
18S ribosomal RNA-AS	5'- GGGACTTAATCAACGCAAGC -3'
FL-*Irs-1* mRNA-S	5'- CTATGCCAGCATCAGCTTCC -3'
FL-*Irs-1* mRNA-AS	5'- TTGCTGAGGTCATTTAGGTCTTC -3'
Δ5' *Irs-1* mRNA -S	5'- TATGCCAGCATCAGCTTCC -3'
Δ5' *Irs-1* mRNA -AS	5'- TAAAAACGCACCTGCTGTGA -3'
Luciferase-S	5'- TGAGTACTTCGAAATGTCCGTTC -3'
Luciferase-AS	5'- GTATTCAGCCCATATCGTTTCAT -3'
GFP-S	5'- GAAGCGCGATCACATGGT -3'
GFP-AS	5'- CCATGCCGAGAGTGATCC -3'

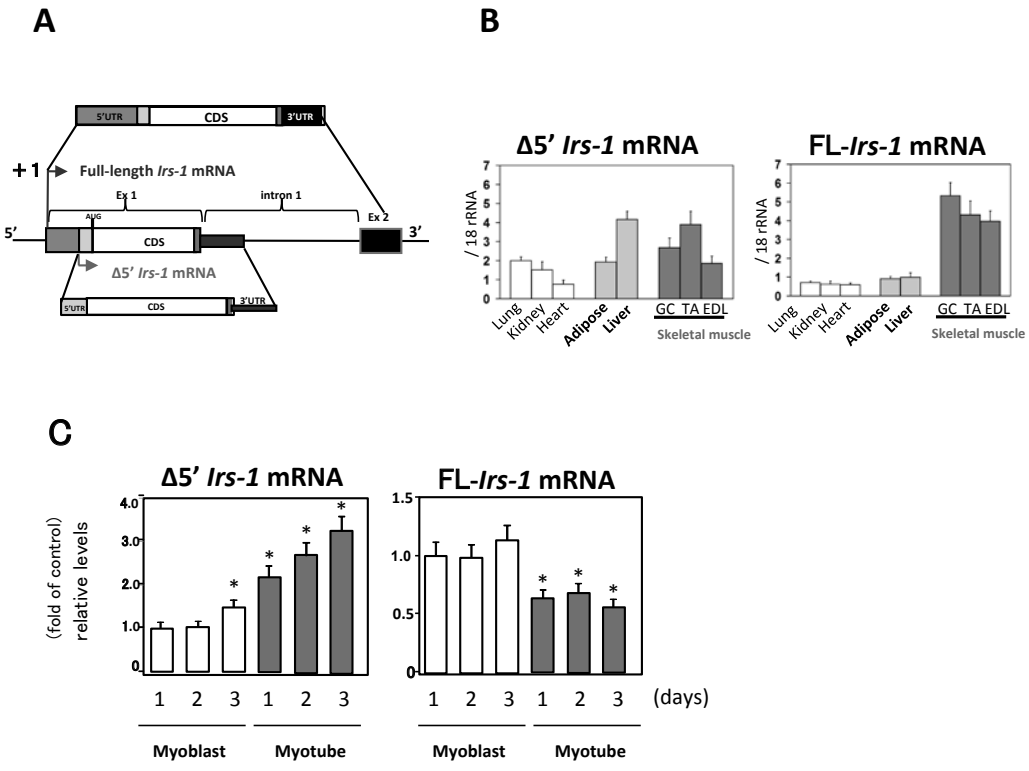

Fig. 1

(A) Schematic representation of two transcriptional variants of mouse *Irs-1* mRNAs (FL- and Δ5'*Irs-1* mRNAs).

(B) Expression profiles of FL-*Irs-1* mRNA and Δ5'*Irs-1* mRNA in the indicated mouse tissues. (GC, gastrocnemius; TA, Tibialis anterior; EDL, Extensor digitorum longus.) Mean ± SD, n=4-6.

(C) Expression levels of FL- *Irs-1* mRNA and Δ5' *Irs-1* mRNA in proliferating C2C12 cells (myoblasts) and differentiating C2C12 cells (myoblast and myotubes). Mean ± SD, n=4. *P < 0.05 versus myoblast day 1.

3 Results

We first searched transcriptional variants of the mouse *Irs-1* gene region using the FANTOM4 database that contains mouse transcriptome data. In the mouse *Irs-1* gene region, there are 19 different transcripts[5]. Among them, we focused on two different *Irs-1* mRNAs (Fig. 1A). One is transcribed from +1 nt transcription start site, thus it contains full-length 5'UTR sequence (FL-*Irs-1* mRNA). The other is transcribed from +534 nt transcription start site, resulting in a short 5'UTR sequence (Δ5'-Irs1 mRNA). Both *Irs-1* mRNAs contain the same protein cording sequence (CDS) of *Irs-1* protein that is fully included in the exon 1.

We examined expression profiles of the two *Irs-1* mRNAs in several mouse tissues (Fig. 1B). Ubiquitous expression of Δ5'-*Irs-1* mRNA was observed in multiple tissues, including skeletal muscle, adipose tissue and liver, known that *Irs-1* mRNA and its protein are highly expressed (Fig. 1B). In contrast, FL- *Irs-1* mRNA was specifically overexpressed in several skeletal muscle tissues (gastrocnemius, tibialis anterior, extensor digitorum longus), compared with other tissues tested (Fig. 1B), suggesting that FL- *Irs-1* mRNA may have skeletal muscle-specific function. In fact, we previously elucidated that FL- *Irs-1* mRNA, but not Δ5'-*Irs-1* mRNA regulates muscle cell differentiation[5].

We then examined expression profiles of the two *Irs-1* transcripts in C2C12 cells under proliferation (myoblasts) and differentiation conditions (myotubes) (Fig. 1C). Expression levels of Δ5'-*Irs-1* mRNA were low in proliferation state, whereas increased to 3 times during differentiation state (Fig. 1C). In contrast, expression levels of FL- *Irs-1* mRNA were high in proliferation state, but decreased to ~50% during differentiation state (Fig. 1C), coincident with our previous observation that FL- *Irs-1* functions under proliferation state[5].

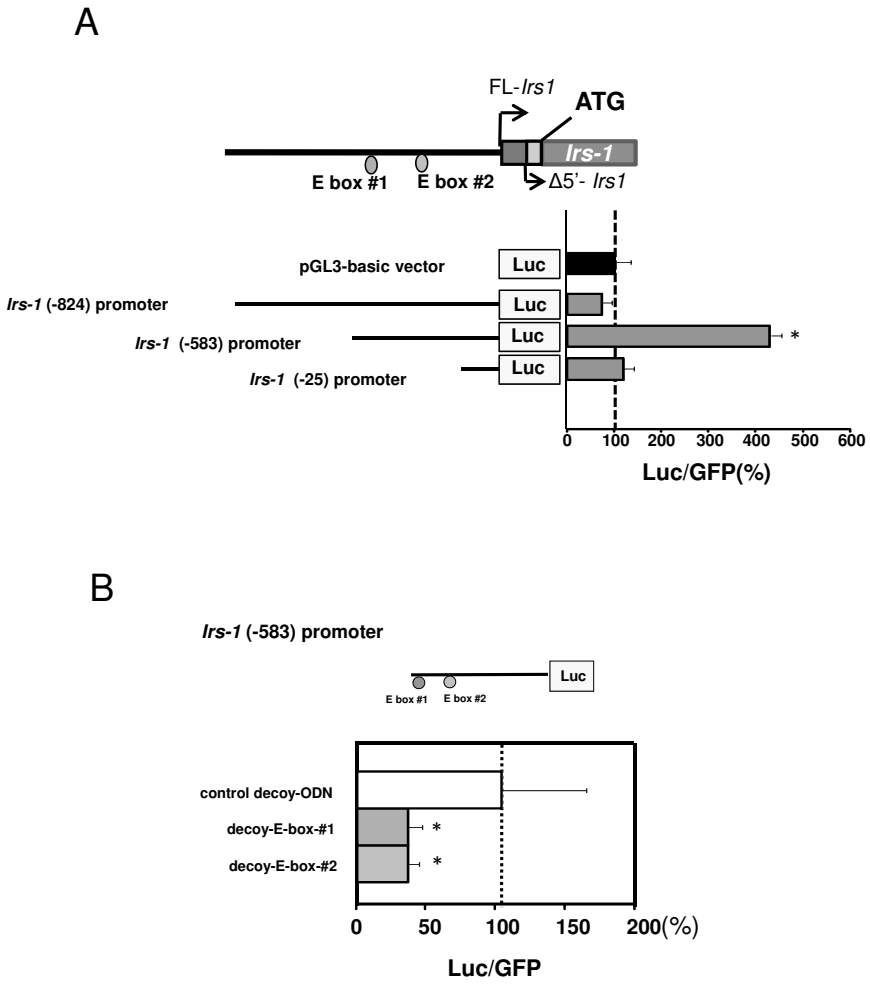

Fig. 2

(A) Determination of *Irs-1* promoter region in C2C12 cells. Schematic structure of the mouse *Irs-1* gene promoter is shown in the top. Predicted binding sites for E-box are indicated by circles. Schematic representation of luciferase reporter constructs containing several *Irs-1* promoter sequences are shown. Mean ± SD, n=3-4. *P < 0.05 versus pGL3-basic vector.

(B) Effect of inhibition of E-box #1 and #2 sites on promoter activity of FL-*Irs-1* mRNA. C2C12 myoblasts were transfected with p*Irs-1* (-583) reporter construct in combination with the indicated promoter decoy-ODN. Mean ± SD, n=4-5. *P < 0.05 versus control decoy-ODN.

We then examined transcriptional regulation of FL-*Irs-1* mRNA in C2C12 myoblasts. To determine the promoter region of FL-*Irs-1* mRNA, we used promoter constructs fused to luciferase reporter gene containing three different 5'-flanking sequence of the mouse *Irs-1* gene, p*Irs-1* (-824) promoter, p*Irs-1* (-583) promoter and p*Irs-1* (-25) promoter (Fig. 2A). The promoter activity was observed in the p*Irs-1* (-583) construct. By contrast, the activity using the p*Irs-1* (-824) or the p*Irs-1* (-25) construct was similar to negative control (empty pGL3-basic). These results indicated that the promoter regions (-583 to -26) contained transcriptional activating element, while the promoter region (-824 to -583) contained transcription suppressive element.

We then searched in silico the canonical binding sites (E-box) of muscle-specific transcription factors (MyoD family), using TransSearch program. The activating region (-583 to -26) contained two E-box sites (Fig. 2A). To determine which E-box site regulated the promoter activity, we used two E-box decoy oligonucleotides (decoy-E-box #1, #2) that inhibit binding of transcription factors to the E-box site (Table.2). When using p*Irs-1* (-583) construct, decoy-E-box #1 and #2 remarkably decreased the promoter activity (Fig. 2B). This finding suggests that E-box #1 and #2 activated FL-*Irs-1* mRNA transcription.

Table 2 Oligo nucleotides

Name	Sequence
E-box #1-S	ACggccaggggtgGA
E-box #1-AS	TCcacccctggccGT
E-box #2-S	TCcgtcacgtgtttTG
E-box #2-AS	CAaaacacgtgacgGA

4 Discussion

Previous studies have shown that IRS-1 protein was highly expressed in skeletal muscle cell and essential in their proliferation and differentiation[5,8]. It has been considered that IRS-1 protein is derived from single transcript in skeletal muscle cells. In this study, we found the existence of a unique transcriptional variant of *Irs-1*mRNA (FL-*Irs-1* transcript) that was overexpressed in skeletal muscle. In addition, FL-*Irs-1* transcript was mainly expressed in proliferation state of skeletal muscle cells.

We further elucidated transcriptional regulation of FL-*Irs-1* transcript in an E-box-dependent manner. Transcription factors that binds to the E-boxes have been shown to consist of heterodimers between a ubiquitous class A and a tissue-specific class B family member of the basic helix-loop-helix (bHLH) transcriptional activators[2]. The muscle-specific class B members, including MyoD family (MyoD, myogenin, MRF4 and MEF2) activates differentiation-specific genes by binding E-box elements. In this study, we revealed that there were two regulatory E-box sites in the promoter region of FL-*Irs-1* mRNA. These sites positively regulated FL-*Irs-1* mRNA transcription (Fig. 2).

5 Conclusions

A unique FL-*Irs-1* transcript was highly expressed in skeletal muscle tissues and cultured myoblasts. In addition, the transcriptional of FL-*Irs-1* mRNA was regulated by E-box-binding transcription factor(s).

Acknowledgments

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Reference

1 Masuda K, Teshima-Kondo S, Mukaijo M, et al. (2008) A novel tumor-promoting function residing in the 5' non-coding region of vascular endothelial growth factor mRNA. PLoS Med, 5:e94.

2 Amack JD, Paguio AP, Mahadevan MS. (1999) Cis and trans effects of the myotonic dystrophy (DM) mutation in a cell culture model. Hum Mol Genet, 8:1975-1984.

3 Amack JD, Reagan SR, Mahadevan MS. (2002) Mutant DMPK 3'-UTR transcripts disrupt C2C12 myogenic differentiation by compromising MyoD. J Cell Biol, 159:419-429.

4 Cesana M, Cacchiarelli D, Legnini I, et al. (2011) A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell, 147:358-369.

5 Nagano H, Yamagishi N, Tomida C, et al. (2015) A novel myogenic function residing in the 5' non-coding region of Insulin receptor substrate-1 (*Irs-1*) transcript. BMC Cell Biol, 16:8.

6 Araki E, Haag BL Matsuda K, et al. (1995) Characterization and regulation of the mouse insulin receptor substrate gene promoter. Mol Endocrinol. 9:1367-79.

7 Edmondson DG, Olson EN. (1993) Helix-loop-helix proteins as regulators of muscle-specific transcription. J Biol Chem, 268:755-758.

8 Hakuno F, Yamauchi Y, Kaneko G, et al. (2011) Constitutive expression of insulin receptor substrate (IRS)-1 inhibits myogenic differentiation through nuclear exclusion of Foxo1 in L6 myoblasts. PLoS One, 10:e25655