



A new cell-based assay to evaluate myogenesis in mouse myoblast C2C12 cells

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ABSTRACT

The development of the efficient screening system of detecting compounds that promote myogenesis and prevent muscle atrophy is important. Mouse C2C12 cells are widely used to evaluate myogenesis but the procedures of the assay are not simple and the quantification is not easy. We established C2C12 cells expressing the N-terminal green fluorescence protein (GFP) and the C-terminal GFP (GFP1–10 and GFP11 cells). GFP1–10 and GFP11 cells do not exhibit GFP signals until they are fused. The signal intensity correlates with the expression of myogenic markers and myofusion. Myogenesis-promoting reagents, such as insulin-like growth factor-1 (IGF1) and β -guanidinopropionic acid (GPA), enhance the signals, whereas the poly-caspase inhibitor, z-VAD-FMK, suppresses it. GFP signals are observed when myotubes formed by GFP1–10 cells are fused with single nuclear GFP11 cells, and enhanced by IGF1, GPA, and IBS008738, a recently-reported myogenesis-promoting reagent. Fusion between myotubes formed by GFP1–10 and GFP11 cells is associated with the appearance of GFP signals. IGF1 and GPA augment these signals, whereas NSC23766, Rac inhibitor, decreases them. The conditioned medium of cancer cells suppresses GFP signals during myogenesis and reduces the width of GFP-positive myotubes after differentiation. Thus the novel split GFP-based assay will provide the useful method for the study of myogenesis, myofusion, and atrophy.

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1. Introduction

Sarcopenia is a skeletal muscle atrophy observed in aged people [1]. In Japan, almost 20% of old people are disabled due to sarcopenia [2]. Similar muscle atrophy is also observed in a wide variety of situations including cancer cachexia, chronic diseases, and malnutrition [3]. As skeletal muscles are the largest metabolic organs in human body, the reduction of the muscle volumes

significantly deteriorates the physical conditions. Thus the maintenance and the recovery of skeletal muscles are crucial for the quality of life of elderly populations and patients with various diseases. Satellite cells are localized between the sarcolemma and the basal lamina in skeletal muscles and are considered as a major source to regenerate muscle tissues in adult [4]. Therefore it is important to develop the drugs that enhance the proliferation of satellite cells and facilitate their differentiation into myofibers with simultaneous replenishment of themselves. Mouse myoblast C2C12 cells were originally established from injured adult C3H mouse leg muscles [5]. C2C12 cells differentiate into contractile myotubes under the appropriate culture conditions and are widely used as the model of myoblasts.

We launched the screening for new chemical compounds that promote myogenesis in C2C12 cells [6]. We first searched for activators of transcriptional coactivator with PDZ binding motif (TAZ,

Abbreviations: GFP, green fluorescence protein; GPA, β -guanidinopropionic acid; IGF1, insulin-like growth factor-1; LLC, Lewis lung carcinoma; MAFbx, Muscle Atrophy F-Box; MHC, myosin heavy chain; MuRF1, Muscle RING-finger-1; TAZ, transcriptional coactivator with PDZ binding motif; YAP1, Yes-associated protein 1
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also called WWTR1), which is known to enhance myogenesis in C2C12 cells [7]. We subsequently selected the activators that promoted myogenesis in C2C12 cells and focused on one compound, IBS008738. This compound increases satellite cells in the injured skeletal muscles and promotes muscle repair. It also prevents steroid-induced muscle atrophy *in vivo*. These findings validate the C2C12-based screening as a method for the discovery of the drugs that are useful for the therapy of muscle diseases.

In this study, we generated a new C2C12-based assay to find out myogenesis-promoting compounds. Green fluorescence protein (GFP) is composed of eleven β -strands. When GFP is divided into two parts, each part does not show fluorescence. However, if two parts associate with each other and reconstitute the whole protein, the fluorescence is recovered [8–10]. We expressed the first 10 β -strands (GFP1–10) and the last β -strand fused to FLAG-14-3-3 ξ (FLAG-14-3-3-GFP11) in C2C12 cells separately. We expected that if C2C12 cells undergo myogenesis and differentiate into myotubes through myofusion, GFP1–10 and FLAG-14-3-3-GFP11 are expressed in the same cell, form a complex, and show fluorescence. If this is the case, we could monitor myogenesis semi-quantitatively in C2C12 cells without immunostainings. In addition, we fused GFP11 to Yes-associated protein 1 (YAP1). YAP1 is similar to TAZ in the molecular structure [11]. Nevertheless, in contrast to TAZ, YAP1 suppresses myogenesis in C2C12 cells [12]. We selected YAP1, expecting that the cells expressing YAP1 give us the good platform for the screening of myogenesis-promoting compounds. Indeed the effect of IBS008738 was more remarkably detected when YAP1 was used. We further tested various GFP11-fused proteins and a different combination of split GFP proteins and found that the selection of appropriate split GFP proteins is important for the sensitivity of the assay.

2. Materials and methods

2.1. DNA constructs

pcDNA-V5/HisB-GFP1–10, pcDNA3.1(+) Smac-GFP11-FLAG, and pCneoFHF were described previously [13,14]. The linker (H892, 5'-ggcctctagaagatctacgcgtggtaccgtcgacgcggc-3' and H893, 5'-cgcggccgcgtcgacggtaccacgcgtatgcttctaga-3') was ligated into NotI/MluI sites of pBudCE4.1 (Invitrogen) to generate pBudCE4.2. NheI/NotI fragment from pBudCE4.2 including the elongation factor promoter-1 α was ligated into XbaI/NotI of pQCXIP (Clontech) and then the linker (H3142, 5'-ggccgctcaggttaacaattggatcc-3' and H-3143, 5'-aattggatccaattgtttaactcagc-3') was subcloned into NotI/EcoRI sites to generate pQCXIP EF H892/H893-H3142/H3143 (hereafter named pQCXIP EF). PCR was performed by use of the primers (H2130, 5'-acgcgtcccgccgagcagccgcgcctcaa-3' and H3228, 5'-agatctaaccatgtaagaaagctttcttta-3') on pCMV SPORT human YAP1 (Open Biosystems). The PCR product was digested with MluI/BglII and ligated with the BglIII/XhoI fragment from pcDNA3.1(+) Smac GFP11-FLAG into MluI/Sall of pCneoFHF to generate pCneoFHF YAP1-GFP11. PCR was performed by use of the primers (H-3225, 5'-gctagcaccatggtgagcaaggcgaggag-3', and H-3236, 5'-gtcgactcacttctgttgggtcttctgt-3') on pcDNA-V5/HisB-GFP1–10. The PCR product was subcloned into pTAKN-2 (BioDynamics Laboratory Inc.) to generate pTAKN-2 GFP1–10. NheI/NotI fragments from pCneoFHF YAP1-GFP11 and from pTAKN-2 GFP1–10 were ligated into XbaI/NotI sites of pQCXIP EF to generate pQCXIP EF-FLAG-YAP1-GFP11 and pQCXIP EF-GFP1–10. 14-3-3 ξ cDNA was obtained by RT-PCR on human cDNA from kidney and lung libraries (Clontech) (hereafter described as human cDNA mixtures) with the primers (H-3310, acgcgtatggatgatcagaggatctggtg and H-3311, agatctgaatcgtcacctcgtatgtctgaagt). The PCR product was subcloned into pTAKN-2 to generate pTAKN-2

14-3-3. MluI/BglII fragment from pTAKN-2 14-3-3 and BglIII/XhoI fragment from pcDNA3.1(+) Smac GFP11-FLAG were ligated into MluI/Sall sites of pCneoFHF to generate pCneoFHF 14-3-3-GFP11. NheI/NotI fragment from pCneoFHF 14-3-3-GFP11 was ligated into XbaI/NotI sites of pQCXIP EF to generate pQCXIP EF-FLAG-14-3-3-GFP11. PCR was performed with the following primers and templates; H-3306, 5'-aggatccctcgagcgcgatcacatggtcctgcac-3' and H-3307, 5'-agaattcctcgagtcactgtcgtcgtcgtc-3' on pcDNA3.1(+) Smac GFP11-FLAG; H-3399, 5'-gggaggggcgatccctcgagcgcgatcacatggtc-3' and H-3400, 5'-agaattcgatcactgtcgtcgtcgtccttcta-3' on pGL3 (Promega) for firefly luciferase; H-3357, 5'-agcggccgcacatgctcctagccaccatgacttc-3' and H-3358 and 5'-agaattcgatcctgttcatttttgaactcgc-3' on pBIND (Promega) for Renilla luciferase; H-3397, 5'-agctagcggccgcaaggtgaggaactaacatgg-3' and H-3398, 5'-cgctgcaggatccgctccacacataaccaga-3' on pUB6/V5-His (Invitrogen) for blastcidinS resistance (bsr) gene; H-3308, 5'-agcggccgcccagctcaccatggatgatg-3' and H-3309, 5'-aggatccgaagcatttgcggtggacgatgga-3' on human cDNA mixtures for human β -actin; H-3362, 5'-agcggccgcacgcgtgccaccatgccattcgtaa-3' and H-3363, 5'-agaattcgatcccttctggtggcggtatcatgtc-3' on human cDNA mixtures for human creatine kinase muscle; H-3413, 5'-agctagcccaacatggtgagcaaggcgaggagc-3' and H-3414, 5'-agcgcggccgtcgtacttactgtcgtcatcgtcc-3' on pMX_ER_Lib [15]; and H-3415, 5'-agaattcgtaaagtattatcggtcgtcgtccc-3' and H-3416, 5'-agcggccgttacagctcgtccttcttgatcag-3' on pMX_ERTS_Dec. The first PCR product was digested with NotI/EcoRI and ligated into NotI/EcoRI of pQCXIP to generate pQCXIP GFP11. The next five PCR products were digested with NotI/BamHI and ligated into the same sites of pQCXIP-GFP11 to generate the retroviral expression constructs of firefly luciferase, Renilla luciferase, bsr gene product, human β -actin, and human creatine kinase muscle fused to GFP11. The PCR product obtained from pMX_ER-Lib was digested with NheI/NotI and ligated into XbaI/NotI sites of pQCXIP-EF to express the N-terminal 1–157 amino acids of GFP fused to the N-terminus of DnaE (the splicing protein of *Synechocystis* sp. PCC6803). The PCR product obtained from pMX_ERTS_Dec was digested with EcoRI/NotI and ligated into the same sites of pCneoFHF to express the C-terminal 158–238 amino acids of GFP to the C-terminus of DnaE. The NheI/NotI fragment was isolated from this vector and ligated into XbaI/NotI sites of pQCXIP-EF for the expression of GFP (1–157)-DnaE and DnaE-GFP(158–238).

2.2. Viral infections

Retrovirus expression vectors were co-transfected with pCL10A.1 (Clontech) in HEK293 cells to generate retrovirus vectors.

3. Reagents and antibodies

Anti-FLAG, anti-GFP, anti-myosin heavy chain (MHC), anti-myogenin, and anti-MyoD antibodies were described previously [6]. The following reagents were obtained from commercial sources; β -guanidinopropionic acid (GPA), (Sigma G6878); z-VAD-FMK (Peptide Institute Inc. 3188-v); peroxidase-conjugated anti-mouse secondary antibody (Cappel); NSV23766 (Santa Cruz sc-204823); and IGF1 (Sigma I8779). IBS008738 was described previously [6].

3.1. Cell cultures

C2C12 cells were cultured at 2.4×10^5 /12-well plate in the growth medium (DMEM containing 10% FBS) and differentiated in the C2C12 differentiation medium containing DMEM and 2% horse serum (Invitrogen). C2C12 cells were infected with retrovirus vectors to generate C2C12 cells expressing GFP1–10, various

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