論文の内容の要旨

Development of an Assay Method for Evaluating Cell Fusion Events Using Split Luciferase Reconstitution Techniques
（スプリットルシフェラーゼ再構成技術を用いた細胞融合評価法の開発）

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1. Introduction

Myogenesis-promoting chemicals are an important source of new pharmaceuticals for the treatment of skeletal muscle atrophy that impairs quality of life. Development of new drugs to treat such muscle wasting requires a robust and quantitative screening method that detects myogenesis. As myocyte fusion is a key step in myogenesis to generate functional skeletal muscles, a widely used approach to estimate myocytes fusion is the use of the fusion index, which is a ratio of the number of nuclei inside the myotubes to the total number of nuclei. However, because detection and counting of nuclei under a fluorescence microscopy are time consuming, this index is not suited for high-throughput screening for myogenesis-promoting compounds. This shortcoming can be overcome by simple fluorescence-based analysis, which correlates myogenesis extent with fluorescence intensity. However, the fluorescence approaches generally have difficulty in screening of chemical libraries: a number of library members possibly show fluorescence, generating high-background signals that disrupt precise estimation of myogenesis. Therefore, a new myogenesis-detection approach must be devised for high-throughput screening of myogenesis-promoting compounds without using microscopy or fluorescence detection.

Herein, we describe a novel bioluminescence-based assay system for evaluating myogenesis, aimed at high-throughput screening of chemical compound libraries to identify myogenesis-promoting chemicals. In this system, a pair of genetically encoded probes based on a protein trans-splicing and

split luciferase reconstitution techniques was designed. C2C12 myoblast cells expressing either of the probes were prepared and cultured in the same cultivation dishes. Fusion of the cells that occurs in the myogenesis process results in encounter of the probes to each other in the cytoplasm. Then the cell fusion was detected from the luminescence of reconstituted Fluc generated through protein trans-splicing reactions. Using this assay system, we performed high-throughput screening for a chemical-compound library and identified two chemical compounds, Imatinib and Doxazosin mesylate, as myogenesis-promoting agents.

2. Results and discussion

2-1. The scheme of the probe for detecting the cell fusion

Firefly luciferase (Fluc) and an intein of DnaE derived from the cyanobacterium *Synechocystis* sp. strain PCC 6803 were used to design a pair of the probes. Fluc was split at the position between 415 and 416 amino acids, producing N-terminal and C-terminal fragments: FlucN and FlucC, respectively. The split DnaE inteins comprise N-terminal 123 amino acids (DnaEn) and C-terminal 36 amino acids (DnaEc). One probe is the FlucN fused with the DnaEn (N-probe), while the other probe is the DnaEc fused with FlucC (C-probe). **(Fig. 2-1A)** Then, two stable cell lines that express either N-probe or C-probe were established based on a C2C12 myoblast cell line (N-cell and C-cell, respectively). N-cell and C-cell were mixture to induce the differentiation and myogenesis. Thus, cell fusion between the N-cells and C-cells during the myogenesis allows the N-probes to encounter the C-probes in the cytoplasm, triggering a protein trans-splicing reaction by DnaEn and DnaEc to connect FlucN with FlucC via a peptide bond between the serine (S) and cysteine (C) and form reconstituted Fluc. **(Fig. 2-1B)**

2-2. Characterization of the N-cells and C-cells.

The N-cell and C-cell were developed by respectively introducing the N-probe (N-cell) and C-probe (C-cell) into the C2C12 cell. We evaluated them from the aspects of the ability in forming myotubes, the expression of corresponding probe and the production of luminescence before and after cell differentiation.

The results of evaluation were as follows. The N-cells and C-cells were undifferentiated initially but differentiated and fused into myotubes after the induction of differentiation. The N-probes were expressed stably in the N-cells and the N-C cell mixture. And the reconstituted Fluc was also observed in the mixed samples from Day 4. However, the C-probes were degraded by proteasome degradation pathway when the probe existed alone in the C2C12 cells (C-cells), whereas the C-probe was stabilized through the interaction with the N-probe when the N-cell fuses to the C-cell. A luminescence signal was not detectable from the N-cells and C-cells neither before or after differentiation, or from the mixture of N-cells and C-cells before differentiation. But, an obvious luminescence signal was obtained from the N-C cell mixture after differentiation.

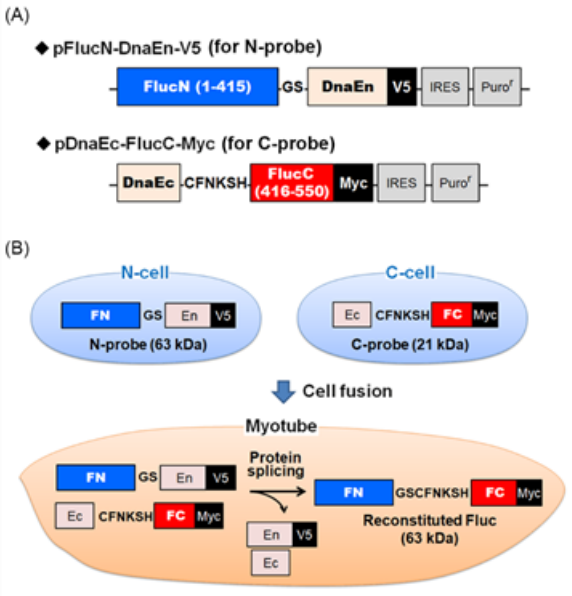

Fig. 2-1. Design of the myogenesis assay system.
(A) The schematic of the retroviral expression vectors for the N-probe and C-probe. The vectors also include puromycin resistance gene. (B) The principle for monitoring the cell fusion. The abbreviations are defined in the main text except FN, FC, En and Ec that respectively indicate FlucN, FlucC, DnaEn and DnaEc

2-3. Correlation of luminescence intensity with differentiation and cell fusion.

To evaluate myogenesis extent, the luminescence signal generated from the mixture of N-cells and C-cells should correlated with the frequency of cell fusion. Therefore, the correlation between the luminescence intensity and the cell fusion frequency was investigated. When the luminescence intensity was show against the fusion index according to the differentiation days, the luminescence intensity showed a linear correlation with fusion index **(Fig. 2-9C)**. The results indicated the use of N-cell and C-cell as an indicator of cell fusion in myogenesis with comparable performance of the fusion index.

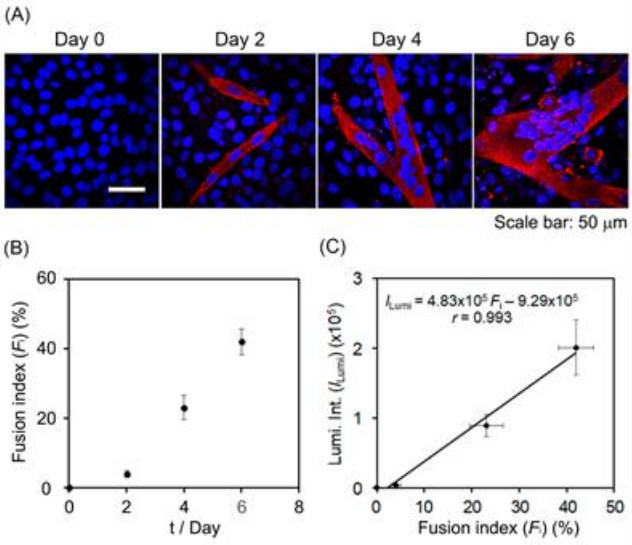

Fig. 2-9. Correlation between luminescence intensities and cell fusion frequency. (A) Immunofluorescence analysis of the mixture of N-cells and C-cells on varying differentiation days. The nuclei (blue dots) were stained with Hoechst 33342; myotubes (red) were labeled with anti-MHC antibody; (B) Fusion index (± SD, n = 3) calculated from the immunofluorescence images on varying differentiation days. (C) Correlation between the luminescence intensity (± SD, n = 3) and the fusion index (± SD, n = 3) 1 mmol L^{-1} D-luciferin was added and luminescence intensities were counted for 30 s.

2-4. The 96-well plate-based assay using the N-cell and C-cell for evaluating myogenesis-promotion by chemical compounds

To assess the applicability of the present system in estimation of the drug efficacy for myogenesis induction in a 96-well plate scale, the progression of cell fusion upon treatment with myogenesis promoters, β-guanidinopropionic acid (GPA, 1.0 mmol L^{-1}) and Insulin-like growth factor 1 (IGF-1, 10 ng mL^{-1}) and an inhibitor, tumor necrosis factor alpha (TNFα, 20 ng mL^{-1}) were assessed.

The imaging data **(Fig. 2-10A)** show the promotion of myotube formation by GPA and IGF-1 while the inhibition by TNFα as expected. And then, the mixture of N-cells and C-cells were cultivated on a 96-well plate to induce the differentiation in the presence of each chemicals, respectively, and the luminescence intensity was counted well by well for 1 s per well. As a result, the decrease/increase in luminescence intensities for the TNFα, the IGF-1, and the GPA groups were consistent with the effects of these agents **(Fig. 2-10B)**.

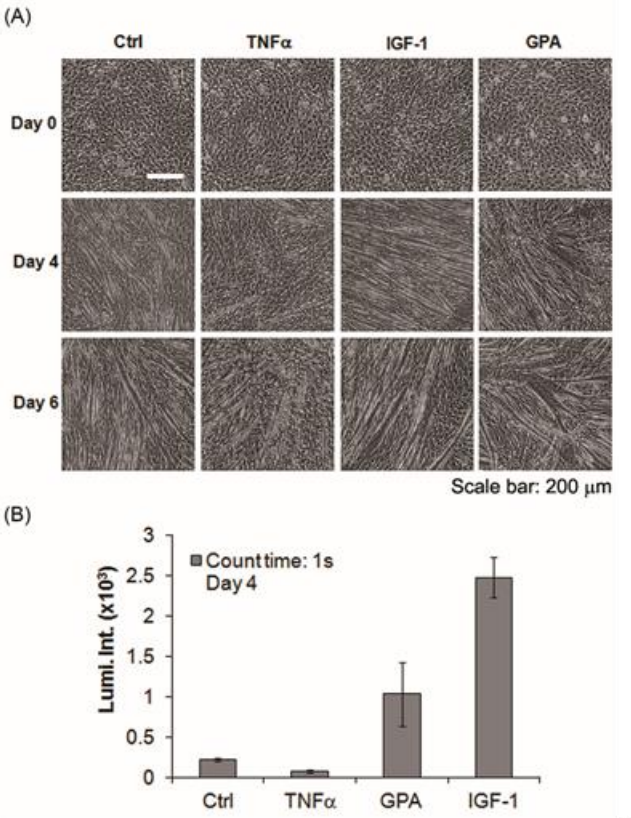

Fig. 2-10. Chemical compound-induced myogenesis inhibition and promotion of C2C12 cells. (A) Myotubes formed in the mixture of N-cells and C-cells treated with TNFα, IGF-1 or GPA; (B) Luminescence intensities (± SD, n = 10) from the mixture of N-cells and C-cells treated with TNFα, IGF-1, and GPA. 0.2 mmol L-1 D-luciferin was used for luminescence measurement.

2-5. Evaluation of the 96-well plate-based assay for a high-throughput screening of myogenesis-promoting chemical compounds

To validate the robustness and quality of the system in the 96-well plate-based assay, Z'-factor was determined. In the determination, DMSO (0.1%) and IGF-1(10 ng mL^{-1}) were used as negative and positive controls, respectively. As a result, a Z'-factor of 0.44±0.05 (Mean±SD) were determined for the 96-well plate-base assay. As the value was in the range of acceptance criterion (Z'- factor ≥ 0.4), the present system showed a sufficient robustness for 96-well plate-based assay to identify chemical compounds that promote myogenesis with the same efficacy as IGF-1.

2-6. Discovery of Imatinib and Doxazosin mesylate for their promoting effects on myogenesis by screening library chemical compounds by using the present 96-well plate assay.

The 96-well microtiter plate-based assay was applied to a high-throughput screening of myogenesis-promoting compounds in 1,191 pharmacologically proven bioactive small molecules (Prestwick Chemical). As a result, two hits compounds, Imatinib and Doxazosin mesylate, were obtained. **(Fig. 3-2)** In addition, dose-dependent effects of Imatinib and Doxazosin mesylate on myogenesis of C2C12 cells were investigated by the present assay as well as the conventional evaluation of fusion index. Two results were consistent that the optimum concentration for promoting the myogenesis of C2C12 cells was ascertained to be 6.0 μmol L^{-1} for Imatinib and 4.0 μmol L^{-1} for Doxazosin mesylate, respectively.

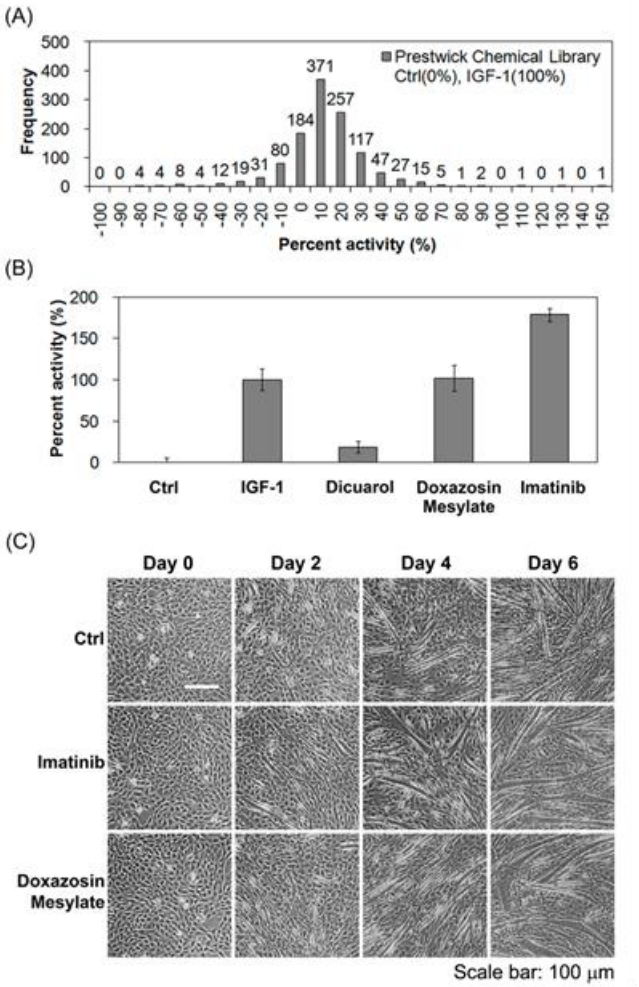

Fig. 3-2. Screening and discovery of myogenesis-promoting compounds from the chemical library. (A) Distribution of the percent activities measured for 1191 chemical compounds. (B) Percent activities (± SD, n = 3) confirmed for three "hit" compounds; (C) Myotubes formed in the mixture of N-cells and C-cells on varying differentiation days under the treatments of Imatinib and Doxazosin mesylate.

3. Conclusion

In this study, a robust assay that enables the detection of cell fusion in myogenesis process was developed using protein *trans*-splicing based luciferase reconstitution technique. The assay method allows quantitative assessment of myogenesis through the luminescence intensity. Because the system is sufficiently sensitive for determining myogenesis promotion of C2C12 cells in a 96-well microtiter plate scale, it is applicable for screening myogenesis-promoting compounds in a high-throughput manner. As a proof of consisting of 1,191 drugs, we found imatinib and doxazosin mesylate to possess a myogenesis-promoting activity in C2C12 cells. Our method may be useful for basic myogenesis studies, tissue engineering and drug discovery. The pair of luciferase probes can be used in broad applications to assess cell fusion events including those accompanied with fusion of nuclei.