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An siRNA-based screen in C2C12 myoblasts identifies novel genes involved in myogenic differentiation

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ABSTRACT

Myogenesis is a highly regulated multi-step process involving myoblast proliferation and differentiation. Although studies over the last decades have identified several factors governing these distinct major phases, many of them are not yet known. In order to identify novel genes, we took advantage of the C2C12 myoblastic line to establish a functional siRNA screen combined with quantitative-imaging analysis of a large amount of differentiated myoblasts. We knocked down 100 preselected mouse genes without a previously characterized role in muscle. Using image analysis, we tracked gene-silencing phenotypes by quantitative assessment of cellular density, myotube quantity, myotube morphology and fusion index. Our results have revealed six genes involved in both stages of C2C12 myogenesis and 13 genes specific to the differentiation stage.

These findings prove that our RNAi-based screen could be an efficient tool to detect clear and subtle phenotypes allowing the identification of new myogenic regulators in mammals.

1. Introduction

The completion of several genome sequences along with technological advances have tremendously accelerated and renovated the tools to identify new genes and to study their function. The functional analysis of each of the thousand of protein-coding genes provided by genome sequencing is a major goal but remains a significant technical challenge [1]. Several genome-wide mutagenesis strategies have been applied in mammals, in order to analyse gene function in a genome-scale manner [2]. These strategies include two complementary approaches: (i) the “phenotype-driven approach” such as ethyl-nitrosourea (ENU) mutagenesis screen [3], which has been successfully used for generating large numbers of new mutants for systematic studies of mammalian gene function [4–6] and for the identification and functional characterization of hundreds of disease-related genes in mice [7], (ii) the “gene-driven approach” which is a high-throughput method used to introduce insertional mutations across the mammalian genome [8]. Several knockout programs were developed to mutate all protein-encoding genes in mouse embryonic stem cells using a combination of gene trapping and gene targeting techniques to generate mice harboring the corresponding genetic changes [9,10]. To accelerate progress towards the genetic analysis of all mammalian genes, large-scale mutagenesis [11], knockout [12,13] and phenotyping [14,15] consortia were established worldwide to generate a wealth of genomic information which are made publicly available to support diverse research efforts, related to understanding mammalian biology and human diseases [16–19]. Despite their strengths, phenotype- and gene-driven approaches remain an important challenge and an experimentally tedious task [20].

Genomic resources such as transcriptomic and proteomic data enable both the simultaneous measurement of the expression of multiple genes and the identification of novel genes or proteins that contribute to the regulation of cellular processes. For instance, to identify new genes and to investigate the global gene expression program of muscle development, several meta-analysis of transcriptomic [21,22], proteomic [23–25] and chromatin immuno-precipitation [26,27] datasets have been performed especially in *in-vitro* mammalian systems. Based only on gene expression patterns, the novel genes identified in genomic resources need further experiments to explore their functions.

The field of functional genomics has been re-energized by the discovery of the RNA interference (RNAi) pathway. RNAi is a regulatory mechanism using small RNAs as specificity factors, recognizing target sequences by base-pairing interactions inducing target mRNA destruction or translation repression [28]. RNAi is currently a valuable gene-silencing tool both in cell culture and in living organisms [29],

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also used for large-scale screens that can systematically knockdown every single gene within a genome in a relatively short period of time [30].

Driven by genomic sequences, RNAi silencing has been used in high-throughput screening aiming at the identification of muscular-specific genes in lower eukaryotes such as *Drosophila and worms* [31–33]. Currently, aside from some RNAi screening in cell transplant–based models of cancer in mouse [34], the *in-vivo* high-throughput screens seem to be difficult to practice generally in mammals and especially in muscle mostly because of low efficiency and high expense [30]. Nevertheless, high-throughput RNAi screens could be used on mammalian primary cultures [35] and cell lines [36] such as C2C12 myoblasts [37,38] which is often used for deciphering the basic molecular mechanisms of muscle-related biological processes. Differentiation of C2C12 myoblasts *in vitro* can easily recapitulate the different steps of *in-vivo* myogenesis [39], a multi-step process enabling generation of muscle tissue during embryonic development and postnatal life [40]. The major myogenic phases, proliferation, differentiation and fusion of myoblasts into myotubes, are well orchestrated by the myogenic regulatory factors and the molecules regulating them *via* different signalling cascades [41].

Despite all the knowledge acquired on the molecular mechanisms regulating myogenesis in mammalian pre- and postnatal development, many of the genes involved are still unknown. In the present study, we have used a newly designed protocol based on a cellular system (C2C12 myoblasts), RNAi technology and quantitative image analysis to develop an siRNA-mediated functional screen, aiming identification of novel genes involved in the myogenesis process. We applied the screen on 100 preselected genes, which were transiently knocked down in proliferating and differentiating C2C12 cells. Phenotypic analysis and gene classification show that six genes are involved during both stages of the C2C12 myogenesis and 13 genes are implicated only during the differentiation stage.

2. Materials and methods

2.1. Cell line

C2C12 cell line (ATCC-CRL-1772) is a subclone produced through serial passages of myoblasts cultured from the thigh muscle of C3H mice after a crush injury [42]. They readily proliferate in high-serum conditions and differentiate and fuse in low-serum conditions, forming contractile myotubes and producing characteristic muscle proteins. C2C12 cells were incubated (at 37 °C in a 5% CO_2 in air atmosphere) during proliferation in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (V/V) heat-inactivated foetal calf serum (FBS), 50 units mL^{-1} penicillin and 50 μg mL^{-1} streptomycin (Growth Medium, GM). To induce C2C12 differentiation, cells at 80% confluence were shifted to DMEM containing 2% (V/V) horse serum (Invitrogen) (Differentiating Medium, DM). The medium was changed every 48 h.

For the screen, C2C12 cells were not directly seeded in the well but on a sterile 1.8 cm^2-coverslips placed at the bottom of the well and covering nearly half of its surface. Cells were seeded at 4.10^4 cells/cm^2 in 1 mL GM for 24 h. Next day, cells were shifted to DM and cultivated for 144 h. During this differentiation time, cells were transfected twice by siRNA (at 0 and 48 h) and medium was changed three times (48, 96 and 120 h). At 144 h, the coverslips with cells were recovered for immuno-staining.

2.2. siRNA Transfection

All siRNAs used in our study are mouse-specific siRNAs designed and produced by Qiagen, delivered in C2C12 cells with Hiperfect transfection reagent (Qiagen) and target either coding sequence or 3'UTR of the mRNA to be knocked down. 100 genes were silenced in 10 groups of 10 genes with two siRNAs for each gene in three technical replicates (Supplementary data, Table S1). For each group of 10 genes, cells transfected with one siRNA (3'alexafluor647, QIAGEN, AATTCTCCGAACGTGTCACGT) without any mouse target as well as untransfected cells were used as controls. The efficiency of siRNA delivery and silencing was tested by RT-PCR and cell viability assays. We verified that the introduction of these synthetic siRNAs into C2C12 cells can trigger highly efficient gene silencing through degradation of the target mRNA (Supplementary data, Fig. S1). C2C12 transfection was performed according to the manufacturers' protocol (Fast-forward transfection of adherent cells, Qiagen). Briefly, siRNAs were diluted at final concentration of 5 nM in 100 μL of culture medium without serum containing 3 μL of Hiperfect transfection reagent. After 10 min incubation at room temperature, the transfection complex was added drop-wise onto cells with fresh medium. Plates were swirled gently, to ensure uniform distribution of the transfection complex, and incubated under normal conditions.

2.3. Immuno-staining

Cells were washed three times in PBS 1X, fixed during 15 min in 4% paraformaldehyde, washed again three times in PBS 1X and permeabilized with HEPES/Triton X-100 buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl2, 3 mM MgCl2 and 0.5% Triton X-100 pH7.4). Then, cells were blocked for 1 h at room temperature in blocking buffer (10% goat serum, 1% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS). After a quick wash in 1X PBS containing 0,2% BSA, cells were incubated overnight at 4 °C with primary antibody anti-MyHC (Monoclonal Anti-Myosin skeletal, Fast, Clone My-32, Mouse Ascities Fluid, M4276, Sigma-Aldrich) diluted (1/400) in 1X PBS containing 1% BSA. After two washes in BSA 0.2% Tween-20 0.1% PBS 1X, coverslips were incubated for 15 min at 37 °C with secondary antibody conjugated to a fluorescent dye (Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L), Invitrogen) diluted (1/1000) in BSA 1% PBS 1X. The staining was completed with three washes in BSA 0,2% PBS 1X Tween-20 0,1%, incubation for 5 min at room temperature in DAPI (1 mg/mL diluted in PBS 1X) and three washes in PBS 1X. Coverslips were finally placed back to front on slides with the mounting solution (Mowiol 4–88 12%, glycerol 24%, tris HCL pH8 0,125 M), sealed with nail polish and stored at 4 °C in the dark.

2.4. Image acquisition and analysis

Images were acquired and analyzed with the automated Leica DMI6000B inverted epifluorescence microscope using the MetaMorph software (Molecular Devices, Sunnyvale, USA). Per coverslip, automated 100 to150 fluorescent images (DAPI or Alexa Fluor 488) were taken in 100X magnification with the aim to reconstruct a large image of the whole scanned zone, the surface of which can reach 1-cm^2. Alexa Fluor and DAPI acquisition parameters were, respectively: wavelengths spectrum 499–519 nm and 345–455 nm, exposure time 1000 ms and 50 ms and light intensity 150. DAPI and Alexa Fluor fluorescence quantifications were done on the corresponding reconstructed images, it consists of the percentage of fluorescent area compared to the whole scanned zone. The total number of nuclei and the number of nuclei inside myotubes were counted, on a section of the overlaid reconstructed images, using ImageJ software (version 1.48q) [43] (http://rsbweb.nih.gov.gate1.inist.fr/ij/). The fusion index of C2C12 cells was calculated as the ratio of the number of nuclei in myotubes to the total number of nuclei. The ImageJ software was also used to assess myotubes size by measuring length and width of 30 myotubes from 15 randomly chosen fields of the Alexa Fluor 488 reconstructed images.