Use of A Randomized Hybrid Ribozyme Library for Identification of Genes Involved in Muscle Differentiation

Renu Wadhwa‡, Tomoko Yaguchi‡, Kamaljit Kaur‡, Eigo Suyama‡, Hiroyuki Kawasaki§, Kazunari Taira‡§, and Sunil C. Kaul‡¶

From the ‡Gene Function Research Center, National Institute of Advanced Industrial Science & Technology (AIST), 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan and the §Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, Hongo, Tokyo 113-8656, Japan

The abbreviations used are: siRNA, small interfering RNA; Rz, ribozyme

¶To whom correspondence should be addressed: Sunil C. Kaul, Gene Function Research Center, National Institute of Advanced Industrial Science & Technology (AIST), 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan Tel: +81 29 861 6713; Fax: +81 29 861 2900. E-mail: s-kaul@aist.go.jp
We have employed hybrid hammerhead ribozyme based gene discovery system for identification of genes functionally involved in muscle differentiation using in vitro myoblast differentiation assay. The major muscle regulatory genes (MyoD1, Mylk, Myosin, Myogenin and Myf5) were identified endorsing the validity of this method. Other gene targets included tumor suppressors and cell cycle regulators (p19ARF and p21WAF1), FGFR-4, fibronectin, Prkg2, Pdk4, fem and 6 novel proteins. Functional involvement of three of the identified targets in myoblast differentiation was confirmed by their specific knockdown using ribozymes and siRNA. Besides demonstrating a simple and an effective method of isolation of gene functions involved in muscle differentiation, we report for the first time that overexpression of fem, a member of sex-determining family of proteins, caused accelerated myotube formation and its targeting deferred myoblast differentiation. This functional gene screening is not only helpful to understand the molecular pathways of muscle differentiation but also to design molecular strategies for myopathological therapies.

**Keywords:** Randomized ribozyme library, Muscle differentiation, C2C12 differentiation assay, p21WAF1, p19ARF, fem
Ribozymes (Rz) are small RNA molecules that catalyze the hydrolysis of specific phosphodiester bonds of RNA strands with which they form base pairing and act as specific molecular scissors providing a very useful tool of studying gene function in vitro and in vivo (1,2). Hammerhead ribozymes (HH-Rz) are among the smallest catalytic RNAs that have been used widely in molecular biology, biotechnology and biomedicine (3-7). These RNAs fold into their active conformation by the binding of metal ions and cleave oligoribonucleotides at specific sites (NUX, where N can be any nucleotide and X can be A, C or U) by mechanisms that have been widely studied in last two decades (8-10). Hammerhead ribozymes recognize the target gene sequence by recognition arms at its 5’ and 3’ ends of its catalytic core. Thus for making a gene specific ribozyme, its recognition arms (7-9 nucleotides each) are designed to include sequence complementary to the target mRNA. Randomization of these 7-9 nucleotides in each arm yields a large variety of ribozymes capable of targeting multiple mRNA substrates. Such pool of randomized ribozymes (library) expressed from a pol III promoter were generated and used as an efficient gene discovery system (11-13). In contrast to the DNA microarrays and yeast two-hybrid systems, ribozyme based screening system has potential to isolate genes directly involved in the phenomenon of interest. In this system, a randomized ribozyme library is expressed into the cells that are screened for a loss or gain of a biological
phenotype. Isolation and sequence analysis of ribozymes from the selected cells depicting the phenotype of interest is performed. The putative target mRNAs for the ribozymes isolated from cells are then predicted by DNA database search.

Successful inactivation of a specific gene \textit{in vivo} by ribozymes depends on the appropriate design of the expression vector, level of expression and its subcellular localization. Various improvements in designing ribozymes and their level of expression have been made in this regard (14-21). Besides, target gene accessibility that plays a major role has been improved by designing the hybrid ribozymes that coupled the cleavage activity of hammerhead ribozymes with the unwinding activity of RNA helicase (22-24). These helicase coupled hybrid ribozymes were far effective in the cleavage of target mRNAs than their conventional counterparts (25, 26). Furthermore, libraries of hybrid ribozymes with randomized binding arms were predicted to have enhanced efficiency for rapid isolation of functional genes. Indeed, these were successfully used for isolation of genes involved in apoptosis (25, 27-30), cell migration and invasion (31, 32) and Alzheimer’s disease (33). In the present study, we have used this novel, simple, efficient and powerful method for isolation of genes involved in muscle differentiation in an \textit{in vitro} cellular model system.

Skeletal muscle differentiation is characterized by muscle-specific gene expression, terminal withdrawal of cells from the
cell cycle, their fusion into multinucleated cells, and assembly of the contractile apparatus (34). A number of studies in recent past have unraveled the role of myogenic transcription factors, chromatin-modifying enzymes in initiation and regulation of muscle differentiation (35, 36). Characterization of signaling cascades and gene functions involved in muscle differentiation is extremely valuable to understand the biology of muscle disorders including Myopathies, Muscular dystrophy, Spinal Muscular Atrophy that involve deregulation of muscle differentiation (37). Mouse myoblast cell line, C2C12, provides an easy and convenient system to study myocyte differentiation. These cells can be differentiated in culture medium containing horse serum and harvested at various time points to characterize the expression profiles of known cell cycle and myogenic regulatory factors by immunoblot analysis (38). We were able to interrupt differentiation of C2C12 cells by introduction of a randomized ribozyme library. Functional involvement of the predicted targets was confirmed by specific knockdown of genes by ribozymes or siRNA.

We demonstrate (i) the isolation of key regulators of muscle differentiation by the Rz-mediated functional gene discovery system validating the worth of this gene discovery system, (ii) the functional involvement of the tumor suppressor genes (p19ARF and p21WAF1) in muscle differentiation implicating the significance of cell cycle regulation, and (iii) a novel gene (fem1) function involved in muscle differentiation.
MATERIALS AND METHODS

Construction of Randomized Hammerhead Ribozyme Libraries-
Chemically synthesized oligonucleotides encoding ribozyme sequences with randomized substrate-binding arms and a pol III termination sequence were converted to double-stranded sequences by PCR as described previously (27). After digestion with Csp45 I and Kpn I, the fragments were cloned downstream of the tRNA promoter in pUC-dt and ribozymes were transcribed in vitro using AmpliScribe T7 transcription kit (Epicentre Technologies, Madison, WI) (25, 27). To generate poly(A)-connected Rz-expression vectors, we inserted a poly(A) sequence of 60 nucleotides between the ribozyme and pol III termination sequence (Figs. 1 A and 1 B). In case of Rz-expression retroviral vectors, ribozyme libraries were inserted into EcoR I and BamH I sites in the pMX-puro vector (25).

Cell culture, Infection and Screening for Functional Genes - Mouse myoblasts C2C12 were used for assay. These were cultured in Dulbecco’s modified Eagle’s minimal essential medium (DMEM)-supplemented with 10% fetal bovine serum, penicillin, and streptomycin and fungizone (Life Technologies, Inc.) at 37°C in an atmosphere of 5% CO2 and 95% air in a humidified incubator. Cells were induced to undergo muscle differentiation by culturing in the medium-supplemented with 2% horse serum. pMX-puro/Rz library (8-10 µg DNA per 10-cm dish) was transfected into mouse packaging cells, PLAT-E, using FuGENE 6
Cells were incubated with the DNA mixture overnight at 37°C and then transferred to 32°C for 48 h in normal DMEM medium to allow the packaging of DNA into the infectious viral particles. Culture medium was collected, filtered and used as virus solution after an addition of polybrene (8 µg/ml). C2C12 cells were infected for 16 h followed by selection in puromycin (2 µg/ml)-supplemented medium for 24-48 h. Selected cells were then induced to differentiate. Undifferentiating and dividing cells were isolated from the differentiating nondividing cells by ring isolation. These were expanded and re-subjected to differentiating medium in a second round of selection. Undifferentiating clones were similarly tested through the third round of differentiation. RNA was prepared from undifferentiating clones using Isogen (Invitrogen). Total RNA (2 µg) was used for RT-PCR. It was reverse transcribed using lower primer (5’-TTT TTT TTT TTT TTT TTG GTA C-3’) and MMLV transcriptase (42°C, 90 mins). Reverse transcribed product was subjected to PCR amplification using upper (5’-tcc ccg gtt cga aac cgg gca-3’) and lower primers (940-520-720/30 sec each, 20 cycles). The amplified PCR product (~150 bp) was cloned into a TA-cloning vector (Promega) and sequenced using T7 primer.

**Construction of Gene Specific Ribozymes and siRNA Expression Vectors** - RNA polymerase III driven hammerhead ribozyme expression plasmids for p19ARF and p21WAF1 were made as described (26,39). The
empty vector containing the tRNA sequence but without ribozyme was used as a negative control.

For construction of siRNA expression vectors, U6 promoter vector was used (40). Target sites for siRNA were selected using an algorithm (http://www.igene-therapeutics.co.jp). Sequences of the Rz and siRNA sites chosen for different genes are listed in Table 2. In a typical example, sense oligos were made; bold letters represent the target site sequence. C to T and G to A mutations (shown by lowercase bold letters) were inserted in the sense strand only. 5 µl of 100 µM sense and antisense oligos for a target site were mixed and annealed in 100-150 mM NaCl in a final volume (20 µl) using thermal cycler {99 °C for 2 min; 72 °C to 4 °C slop in 2 h}. Annealed oligos were diluted (1:200) and 2 µl was ligated to a BspM I cut and gel purified pciPur vector (40) using high ligation kit (Takara). Plasmid DNA prepared from the transformed bacteria was sequenced with a vector primer (CAGGAAACAGCTATGAC) for confirmation of the integrity of the cloned DNA fragments.

Analysis of Functional Involvement of Genes during Muscle Differentiation - C2C12 cells were transfected with specific ribozyme or
with siRNA gene knockdown constructs using LipofectAMINE™ PLUS (Invitrogen). Transfected cells were selected in puromycin (2.5 µg/ml)-supplemented medium for 2-4 days and then subjected to differentiation. Vector transfected cells were used as control. Myotube formation was monitored in control and in gene knockdown cells. Expression of muscle specific genes in control and gene-knockdown cells were analyzed by Western blotting with specific antibodies as given below.

**Western Blot Analysis** - Immunoassays were performed as described (26). Anti-myogenin (F5D, Santa Cruz), anti-p21^WAF1^ (C-19, Santa Cruz), anti-V5 (Invitrogen) and anti-actin (Chemicon) antibodies were used. The immunocomplexes formed were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies using ECL PLUS kit (Amersham Biosciences).

**Immunostaining** - C2C12 cells and its derivatives (transfected with either expression or siRNA plasmids for fem1 as indicated) were induced for differentiation by incubating in the growth medium-supplemented with 2% horse serum. Cells were fixed with methanol:acetone (1:1) at various time points and then stained with anti-MHC antibody (Novocastra). Alexa-488-conjugated goat anti-mouse (Molecular Probes) antibody was used as secondary antibody. The cells were examined on a Carl Zeiss microscope.
RESULTS AND DISCUSSION

Abrogation of C2C12 Differentiation with Randomized Hybrid Ribozyme Library - To explore the genes functionally involved in muscle differentiation, C2C12 myoblasts were infected with randomized ribozyme library and subjected to differentiation medium as shown in Fig. 1C. Whereas control (empty vector infected) cells formed full myotubes in 72 to 96 h in differentiation medium (Fig. 2a and c), cells infected with the randomized ribozyme library showed significant abrogation of myotube formation (Fig. 2b and d). Undifferentiating cells that continued to divide formed small colonies against the background of differentiating cells that fused to form myotubes (Fig. 2b). We isolated 96 and 12 undifferentiating clones from the Rz- and empty vector-infected cultures, respectively (Fig. 2). Cells were expanded to 60-70% confluency in 48- and 24-well plates for second and third rounds of selection for undifferentiating clones. We obtained 67 undifferentiating clones from the Rz-infected culture and none from the empty vector-infected culture. Ribozymes were recovered from the undifferentiating cells by RT-PCR and the sequence of these ribozymes was obtained as shown in the scheme in Fig. 1C and described in Material and methods.
Candidate Genes for C2C12 Differentiation

 Known Key Regulators of Muscle Differentiation - By three rounds of selection, we isolated 67 independent undifferentiating C2C12 derivative clones. Ribozyme sequences recovered from these clones matched with 33 different target sites that aligned to 24 different mRNAs (Table 1). Five (MyoD1, Mylk, Myosin, Myogenin and Myf5) out of the twenty-four were the genes specifically expressed in muscles. Myf5 is the first gene to be expressed followed by myogenin, myoD, and myf6 during embryonic muscle development in mice. Its expression is detected in mononucleated myoblasts whereas myogenin and myoD accumulate in mono- and multinucleated myogenic cells (41). MyoD and Myf5 are master regulatory genes for myogenic determination, and myogenin and myosin (muscle structural proteins) are important for terminal differentiation and lineage maintenance (42, 43). Although Mylk (myosin light chain kinase) and Myosin are known to be involved mainly in muscle contractility (44), our data suggests that they may also be involved in muscle differentiation. Based on the characteristics and known function of the isolated genes, their detection strongly validated the use of ribozyme-based functional gene discovery approach.

 Genes Involved in Extra Cellular Signaling: Fibronectin and FGFR4 - Five ribozymes matched to three independent sites on fibronectin. Integrin-mediated cell adhesion to extracellular matrices
provides signals essential for cell cycle progression and differentiation (45). Differences in fibronectin conformations were shown to alter the quantity of bound subunits of integrin and regulate differentiation. Treatment of myoblasts with specific inhibitors of proteoglycan synthesis (sodium chlorate and beta-D-xyloside) substantially affected the deposition and assembly of the extra cellular matrix (ECM) constituents (glypican, fibronectin, and laminin) and meddled their differentiation without affecting the expression of muscle differentiation regulators (MyoD, MEF2A, and myogenin). Treatment of differentiated myoblast with RGDS peptides completely inhibited myogenesis without affecting myogenin expression. Interestingly, antibodies specific for RGD binding site in fibronectin abolished myoblast differentiation (46-48). Based on these data it was concluded that the expression of myogenin is not sufficient to successfully drive muscle formation and that ECM is required to complete the skeletal muscle differentiation process.

Fibroblast growth factors (FGFs) were identified as powerful stimulators of myoblast proliferation and inhibitors of myoblast differentiation in vitro. In chick embryos, most if not all, replicating myoblasts, but not the differentiated muscle cells, express high level of the FGF receptor FREK/FGFR4. It preceded MyoD expression that signals the onset of terminal differentiation suggesting an important role of FGF receptor in muscle differentiation and one of the earliest
molecular markers (49). It was shown that an inhibition of FGFR4, but not FGFR1 signaling affects Myf5, MyoD and myosin heavy chain expression and dramatic loss of limb muscles. Conversely, overexpression of FGF8 in somites promoted FGFR4 expression and muscle differentiation. FGFR4 signaling was denoted as a crucial step in the cascade of molecular events leading to terminal muscle differentiation (50). In light of this information, our screening assay for functional genes is proved to be highly efficient, easy and informative.

Tumor Suppressors and Cell Cycle Regulator Genes - Three of the twenty four target genes were the tumor suppressors and cell cycle regulators (p27, p19ARF and p21WAF1). During muscle differentiation, muscle-specific gene induction and transition of cells from the proliferative stage to form post-mitotic multinucleated myotubes are regulated through highly ordered and temporally separable events. Cell cycle arrest is critical for muscle differentiation and involves the inhibitors of cyclin-dependent kinases (CDKs) (51-53). The level of p27 protein gradually increases with differentiation (54). p19ARF is an upstream regulator of p53 and p21WAF1 (inhibitor of cyclin-dependent kinase). By inhibiting the cyclin-dependent kinase, p21WAF1 regulates the activity of retinoblastoma tumor suppressor protein (Rb); hypophosphorylated pRb binds to E2F and causes G0 cell cycle arrest. p21 expression is also activated by a muscle-specific transcriptional
regulator, MyoD, independent to that of p53 and causes cell cycle withdrawal (55). p21-/- mice display increased cell number and enhanced cell cycle progression of myogenic progenitor cells and impaired muscle differentiation and regeneration (56, 57). Duchenne muscular dystrophy (DMD, decreased muscle cell proliferation phenotype), caused by the absence of dystrophin, involves an increased expression of p21\(^{\text{WAF}}\). Interestingly, an appropriate transient transfection of p21-antisense oligos improved their proliferation (58). In light of these data, isolation of cell cycle regulators as functionally involved in muscle differentiation was justified.

We further tested the functional role of p19\(^{\text{ARF}}\) and p21\(^{\text{WAF}}\) by their specific knockdown using ribozymes (Rz) and siRNA. Target sites for p19\(^{\text{ARF}}\) and p21\(^{\text{WAF}}\) are shown in Table 2. Efficacy of the ribozymes to knockdown their targets (p19\(^{\text{ARF}}\) and p21\(^{\text{WAF}}\)) was examined by exogenous expression of epitope-tagged proteins and Western blotting with tag-specific antibodies. We found that all the five p19\(^{\text{ARF}}\) Rz constructs and the two p21\(^{\text{WAF}}\) Rz constructs could bring down the expression of their respective targets by 30-50% (data not shown). We next traced the formation of C\(_\text{2C12}\) myotubes in control and p19\(^{\text{ARF}}\)-Rz transfected myoblasts and found that 2/5 ribozymes (#19-2 and #19-4) resulted in delayed tube formation (Fig. 3A). This was also accompanied by decrease in myogenin; an established marker for
myotube formation. The p19ARF Rz-derivatives also showed a lower level of p21WAF-1 and myogenin as compared to the vector trasfected control cells. Of note, the effect of different p19ARF Rzs on p21WAF1 and myogenin was well correlated with their effect on differentiation potential; the Rzs that effectively reduced p21 WAF-1 and myogenin constrained myoblast differentiation.

We next targeted p21WAF1 with ribozymes. Interestingly, one of the two p21WAF-1 ribozymes that caused sharp decrease in myogenin resulted in significant delay in myotube formation (Fig. 3B). Whereas control cells formed full myotubes in 60 h, the #21-4 Rz transfected cells showed high population of unaligned myoblasts. A more efficient knockdown of p19ARF and p21WAF1 was attempted with siRNAs (target sites are shown in Table 2). One of the p19ARF siRNA (#19-440) and both of the p21WAF1 siRNA (#21-59 and #21-625) (Fig. 3C) resulted in decreased expression of the respective target genes and myogenin. This was accompanied by significantly delayed myotube formation (Fig. 3D). These data confirmed the functional involvement of p19ARF and p21WAF1 in myoblast differentiation.

A Novel Function of Sex-Determining Family Protein-fem -

*Caenorhabditis elegans* fem genes (fem1, fem2, and fem3) are centrally involved in male sexual development including initiation of spermatogenesis in XX (hermaphrodite) worms, and the entire
spectrum of male differentiation in XO animals (59, 60). Fem proteins are conserved in mice and human suggesting their function in similar pathways. These contain six to seven contiguous copies of a motif (ANK repeats) found in cell cycle regulating proteins (the cdc10 of *S. pombe*, the SWI6 gene of *S. cerevisiae*, the Notch gene of Drosophila, and the lin-12 and glp-1 genes of *C. elegans*) (61). Fem2 sequence is related to protein serine/threonine phosphatases of Type 2C (PP2C) and exhibits magnesium-dependent casein phosphatase activity that is critical for its function in male development in worms (62). Murine homologues fem1a and fem1b are expressed during embryogenesis; the fem1a expression is enriched in adult heart and skeletal muscle and fem1b is highly expressed in adult testis suggesting their unique tissue specific functions (63, 64). Fem proteins are negatively regulated by Tra-2 that prevents male development; a balance between the opposing activities of Tra-2A and fem-3 determines sex-specific cell fates in somatic tissues. Overexpression of fem-3 could overcome the feminizing effect of tra-2 and caused widespread masculinization of XX somatic tissues (65).

In our screen, seven ribozymes that hit three independent sites of murine fem homolog (fem1c) were isolated. We decided to analyze the functional involvement of fem protein in muscle differentiation by its specific knockdown with siRNA. Target sites are shown in Table 2. Cells were transfected with siRNAs and selected in puromycin following which equal number of vector and fem siRNA tranfected cells were
plated. We found that fem-siRNAs improved the proliferation potential of myoblasts (Fig. 4A, cf. b and c with a). We next transfected cells with expression plasmid for fem1 and found that these cells show early tube formation as compared to the control cells (Fig. 4B, cf. c and d with a and b). Transfection of fem1 siRNAs (fem-1000 and fem-2203) caused remarkable delay in tube formation as visualized by phase contrast microscopy (Fig. 4B, cf. e and f with a and b) and immunostaining with anti-myosin heavy chain antibody (Fig. 4C). This was accompanied by decrease in myogenin level (Fig. 4D). In the absence of antibodies specific to fem1, specificity of fem-siRNAs to its target was ensured by exogenous expression of V5-tagged fem and its analysis by anti-V5 antibodies. We found that these siRNAs were highly specific to fem1 and caused up to 55% reduction in the protein level (Fig. 4E). Taken together, we have exposed a novel function of the fem1 protein by employing ribozyme based functional gene discovery system.

**Novel Gene Functions for Muscle Differentiation** - Our screen has identified six novel genes (Gene bank accession numbers: 1700025D19, 2410015A15, 4933405K18, 6820443O06, A630095P14 and A930024E05 (Table 1) putatively involved in muscle differentiation. Further studies on specific knockdown of these genes are warranted. These studies will not only enhance our understanding on the functional biology of muscle differentiation but may also provide information for therapy of muscle diseases.
Taken together, we have demonstrated (i) an effective use of a randomized ribozyme library for identifying genes for muscle differentiation, (ii) the validity of this approach by specific knockdown of some mRNAs, and (iii) the functional involvement of tumor suppressors (p19ARF and p21WAF1) and a sex-determining protein fem1 in muscle differentiation.

We thank M. Miyagishi for help in siRNA vector construction. This work was supported in part by a research grant from AIST, Japan.

REFERENCES
1. Uhlenbeck, O. C. (1987) *Nature* **328**, 596-600
2. Cech, T. R. (2002) *Biochem. Soc. Trans.* **30**, 1162-1166
3. Stage-Zimmermann, T. K., and Uhlenbeck, O. C. (1998) *RNA* **4**, 875-889
4. Rossi, J. J. (1999) *Curr. Opin. Mol. Ther.* **1**, 316-322
5. Michienzi, A., Castanotto, D., Lee, N., Li, S., Zaia, J. A., and Rossi, J. J. (2003) *Ann. N. Acad. Sci.* **1002**, 63-71
6. Sioud, M. (2001) *Curr. Mol. Med.* **1**, 575-588
7. Kato, Y., Kuwabara, T., Toda, H., Warashina, M., and Taira, K. (2000) *Nucleic Acids Symp. Ser.* **44**, 283-284
8. Verma, S., Vaish, N. K., and Eckstein, F. (1997) *Curr. Opin. Chem. Biol.* **1**, 532-536
9. Takagi, Y., Suyama, E., Kawasaki, H., Miyagishi, M., and Taira, K. (2002) Biochem. Soc. Trans. **30**, 1145-1149

10. Goodchild, J. (2002) Expert Opin. Ther. Targets **6**, 235-247

11. Kruger, M., Beger, C., Li, Q. X., Welch, P. J., Trizt, R., Leavitt, M., Barber, J. R., and Wong-Staal, F. (2000) Proc. Natl. Acad. Sci. USA **97**, 8566-8571

12. Welch, P. J., Marcusson, E. G., Li, Q. X., Beger, C., Kruger, M., Zhou, C., Leavitt, M., Wong-Staal, F., and Barber, J. R. (2000) Genomics **66**, 274-283

13. Kawasaki, H., and Taira, K. (2001) Nucleic Acids Res. Suppl., 133-134

14. Koseki, S., Tanabe, T., Tani, K., Asano, S., Shioda, T., Nagai, Y., Shimada, T., Ohkawa, J., and Taira, K. (1999) J. Virol. **73**, 1868-1877

15. Kuwabara, T., Warashina, M., and Taira, K. (2002) J. Biochem. **132**, 149-155

16. Miyagishi, M., Kuwabara, T., and Taira, K. (2001) Cancer Chemother. Pharmacol. **48**, S96-101

17. Kuwabara, T., Warashina, M., and Taira, K. (2000) Trends Biotechnol. **18**, 462-468

18. Sano, M., Kuwabara, T., Warashina, M., Fukamizu, A., and Taira, K. (2002) Antisense Nucleic Acid Drug Dev. **12**, 341-352

19. Sano, M., Kuwabara, T., Nara, Y., Warashina, M., and Taira, K. (2000) Nucleic Acids Symp. Ser. **44**, 203-204

20. Zhao, J. J., and Lemke, G. (1998) Mol. Cell Neurosci. **11**, 92-97

21. Iyo, M., Kawasaki, H., and Taira, K. (2004) Methods Mol. Biol. **252**, 257-265
22. Warashina, M., Kuwabara, T., Kato, Y., Sano, M., and Taira, K. (2001) 
   Proc. Natl. Acad. Sci. USA 98, 5572-5577
23. Kawasaki, H., Warashina, M., Kuwabara, T., and Taira, K. (2004) Methods Mol. Biol. 252, 237-243
24. Akashi, H., Kawasaki, H., Kim, W. J., Akaike, T., Taira, K., and Maruyama, A. (2002) J. Biochem. 131, 687-692
25. Kawasaki, H., and Taira, K. (2002) EMBO Rep. 3, 443-450
26. Wadhwa, R., Ando, H., Kawasaki, H., Taira, K., and Kaul, S. C. (2003) EMBO Rep. 4, 595-601
27. Kawasaki, H., Onuki, R., Suyama, E., and Taira, K. (2002) Nat. Biotechnol. 20, 376-380
28. Kawasaki, H., and Taira, K. (2002) Nucleic Acids Res. 30, 3609-3614
29. Kawasaki, H., Tsunemi, M., Iyo, M., Oshima, K., Minoshima, H., Hamada, A., Onuki, R., Suyama, E., and Taira, K. (2002) Nucleic Acids Res. Suppl. 275-276
30. Onuki, R., Kawasaki, H., Baba, T., and Taira, K. (2003) Antisense Nucleic Acid Drug Dev. 13, 75-82
31. Suyama, E., Kawasaki, H., Kasaoka, T., and Taira, K. (2003) Cancer Res. 63, 119-124
32. Suyama, E., Kawasaki, H., Nakajima, M., and Taira, K. (2003) Proc. Natl. Acad. Sci. USA 100, 5616-5621
33. Onuki, R., Bando, Y., Suyama, E., Katayama, T., Kawasaki, H., Baba, T., Tohyama, M., and Taira, K. (2004) EMBO J. 23, 959-968
34. Taylor, M. V. (2003) *Curr. Biol.* **13**, R964-966

35. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2002) *Curr. Opin. Cell Biol.* **14**, 763-772

36. Pownall, M. E., Gustafsson, M. K., and Emerson, C. P., Jr. (2002) *Annu. Rev. Cell. Dev. Biol.* **18**, 747-783

37. Amack, J. D., and Mahadevan, M. S. (2004) *Dev. Biol.* **265**, 294-301.

38. Shen, X., Collier, J. M., Hlaing, M., Zhang, L., Delshad, E. H., Bristow, J., and Bernstein, H. S. (2003) *Dev. Dyn.* **226**, 128-138.

39. Kato, Y., Kuwabara, T., Warashina, M., Toda, H., and Taira, K. (2001) *J. Biol. Chem.* **276**, 15378-15385

40. Miyagishi, M., and Taira, K. (2002) *Nat. Biotechnol.* **20**, 497-500

41. Rohwedel, J., Maltsev, V., Bober, E., Arnold, H. H., Hescheler, J., and Wobus, A. M. (1994) *Dev. Biol.* **164**, 87-101

42. Wei, Q., and Paterson, B. M. (2001) *FEBS Lett.* **490**, 171-178

43. Franchini, M. (2003) *Recent Prog. Med.* **94**, 478-483

44. Lazar, V., and Garcia, J. G. (1999) *Genomics* **57**, 256-267

45. Bouzeghrane, F., Mercure, C., Reudelhuber, T. L., and Thibault, G. (2004) *J. Mol. Cell Cardiol.* **36**, 343-353

46. Osses, N., and Brandan, E. (2002) *Am. J. Physiol. Cell Physiol.* **282**, C383-394

47. Morla, A. O., and Mogford, J. E. (2000) *Biochem. Biophys. Res. Commun.* **272**, 298-302
48. Garcia, A. J., Vega, M. D., and Boettiger, D. (1999) *Mol. Biol. Cell* **10**, 785-798

49. Marcelle, C., Wolf, J., and Bronner-Fraser, M. (1995) *Dev. Biol.* **172**, 100-114

50. Marics, I., Padilla, F., Guillemot, J. F., Scaal, M., and Marcelle, C. (2002) *Development* **129**, 4559-4569

51. Andres, V., and Walsh, K. (1996) *J. Cell Biol.* **132**, 657-666

52. Adams, G. R., Haddad, F., and Baldwin, K. M. (1999) *J. Appl. Physiol.* **87**, 1705-1712

53. Cabane, C., Englaro, W., Yeow, K., Ragno, M., and Derijard, B. (2003) *Am. J. Physiol. Cell Physiol.* **284**, C658-666

54. Franklin, D. S., and Xiong, Y. (1996) *Mol. Biol. Cell* **7**, 1587-1599

55. Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., and Lassar, A. B. (1995) *Science* **267**, 1018-1021

56. Hawke, T. J., Meeson, A. P., Jiang, N., Graham, S., Hutcheson, K., DiMaio, J. M., and Garry, D. J. (2003) *Am. J. Physiol. Cell Physiol.* **285**, 25

57. de la Serna, I. L., Roy, K., Carlson, K. A., and Imbalzano, A. N. (2001) *J. Biol. Chem.* **276**, 41486-41491

58. Endesfelder, S., Bucher, S., Kliche, A., Reszka, R., and Speer, A. (2003) *J. Mol. Med.* **81**, 355-362

59. Doniach, T., and Hodgkin, J. (1984) *Dev. Biol.* **106**, 223-235

60. Kimble, J., Edgar, L., and Hirsh, D. (1984) *Dev. Biol.* **105**, 234-239

61. Spence, A. M., Coulson, A., and Hodgkin, J. (1990) *Cell* **60**, 981-990
62. Chin-Sang, I. D., and Spence, A. M. (1996) *Genes Dev.* **10**, 2314-2325

63. Ventura-Holman, T., and Maher, J. F. (2000) *Biochem. Biophys. Res. Commun.* **267**, 317-320

64. Ventura-Holman, T., Lu, D., Si, X., Izevbigie, E. B., and Maher, J. F. (2003) *Gene* **314**, 133-139

65. Mehra, A., Gaudet, J., Heck, L., Kuwabara, P. E., and Spence, A. M. (1999) *Genes Dev.* **13**, 1453-1463
LEGENDS TO THE FIGURES

FIG. 1.  **Schematic presentation of randomized ribozyme (Rz) based gene discovery approach.**  Cleavage of the target mRNA with hammerhead ribozyme (HH-Rz) (A).  HH-Rz with randomized arms cloned into a retroviral expression vectors driven by a pol III promoter (B).  Poly A-linked HH-Rz embedded in tRNA and terminator.  Ampicillin and puromycin resistance markers were used for selection in bacteria and mammalian cells, respectively.  Abrogation of myoblast differentiation by randomized ribozyme library, rescue of ribozymes from undifferentiating cells, Rz-cloning, sequencing and target search by Blast analysis (C)

FIG. 2.  **Abrogation of C\textsubscript{2}C\textsubscript{12} differentiation by randomized Rzs.**  C\textsubscript{2}C\textsubscript{12} control (a) and randomized ribozyme library transduced (b) cells cultured in differentiation medium (c and d).  Library transduced myoblasts did not form myotubes.

FIG. 3.  **Myotube formation in control C\textsubscript{2}C\textsubscript{12} (19-0) and their p19\textsuperscript{ARF-Rz} derivatives (19-1 to 5).**  Cells transfected with 19-2 and 19-4 showed delay in myotube formation (upper panel).  These cells also showed decreased level of p21\textsuperscript{WAF1} and myogenin (lower panel) (A).  Myotube formation in C\textsubscript{2}C\textsubscript{12} control (21-0) and p21\textsuperscript{WAF1-Rz} transfected cells.  Cells transfected with Rzs 21-1 and 21-4 showed delay in myotube formation (upper panel) and decreased amount of myogenin (lower panel) (B).  Decreased level
of p19ARF and p21WAF-1 expression in myoblasts transfected with p19ARF and p21WAF-1 siRNAs (C). Cells showing effective knockdown of p19ARF (19-440) and p21WAF-1 (21-59 and 21-625) showed decreased myogenin (C) and delayed myotube formation (D).

FIG. 4. Effect of fem-knockdown and –overexpression on C2C12 differentiation. Increased proliferation of C2C12 myoblasts transfected with siRNA for fem-1 (A). Cells transfected with fem-1 expression plasmid showed enhanced and early myotube formation (B, cf. c with a and b) and the cells transfected with expression plasmid and siRNA for fem-1 showed delayed myotube formation (B, cf. d with e and f). Myosin heavy chain staining of C2C12 cells and their derivatives (fem1-overexpressing and fem1-compromised) after 36 and 72 h of induction of differentiation (C). Cells transfected with siRNA for fem1 showed decreased myogenin (D). Cells transfected with siRNA for fem (1000 and 2203) showed decreased amount of exogenous V5-tagged full-length fem (1-616 amino acids) and its amino-terminus 400 amino acids (1-400). V5-tagged mortalin was used as a negative control and no change in its expression level was seen (E).
**Fig. 1**

A. Diagram illustrating the mechanism of action of a Hammerhead ribozyme. The ribozyme catalyzes the cleavage of substrate RNA.

B. Diagram showing the transcription and cloning of RNA from a specific region of DNA. The process includes the use of a ribozyme and a helper protein to aid in the transcription process.

C. Diagram outlining the process of target validation by Rz and siRNA. The flowchart includes steps for cloning and sequence identification, followed by differentiation medium and target validation.

Legend:
- Hammerhead ribozyme
- Substrate RNA
- Cleaved RNA
- Target cleavage
- Differentiation medium
- Muscle differentiation genes
- Target validation by Rz and siRNA
- Blast search for target identification
- Cloning and sequence of Rz
- Rz-recovery by RT-PCR
Fig. 2
| No. | Rz sequence          | No. Putative target                                    |
|-----|----------------------|-------------------------------------------------------|
| 1   | gcttcatngccgccac    | 4 Myogenic differentiation 1 (Myod1)                  |
| 2   | tcatcatagntctgct   | 3 Myogenic differentiation 1 (Myod1)                  |
| 3   | gcttcatngccgccac    | 3 Myogenic differentiation 1 (Myod1)                  |
| 4   | gacgactttntatgtga   | 3 Myogenic differentiation 1 (Myod1)                  |
| 5   | agacactgtnagcccgac  | 2 Myosin light chain kinase (Mylk)                    |
| 6   | tctcaggenacgctcg    | 3 Myosin heavy polypeptide 4                          |
| 7   | aaaaacgcanactcttgt  | 1 Myosin X mRNA                                       |
| 8   | agcaccctgntcaaccct  | 2 Myoglobin (Myog)                                   |
| 9   | gaggaagtnaactaccc   | 2 Myogenin-related factor 5 (Myf5)                    |
| 10  | ccaagagtngccagct    | 3 Myogenin-related factor 5 (Myf5)                    |
| 11  | gggcacttnntntntttt  | 2 Cyclin-dependent kinase inhibitor 1B (p27)         |
| 12  | tttctcaggnacagctga  | 4 Alternative reading frame exon 1, p19 ARF           |
| 13  | tgtgtgnttaatttattt  | 3 Cyclin-dependent kinase inhibitor 1A (p21)         |
| 14  | agcactgtnagcctctg   | 3 Fibroblast growth factor receptor 4 (Fgfr4)        |
| 15  | agtaagagacactctgc   | 2 Fibronectin 1                                       |
| 16  | cattctgcnacagccat   | 1 Fibronectin 1                                       |
| 17  | tataacctctctctaacct | 2 Fibronectin 1                                       |
| 18  | gacaactgtnaactecgt  | 3 Protein kinase, cGMP-dependent, type II (Prkg2)     |
| 19  | enacagggnaggggac    | 2 Pyruvate dehydrogenase kinase 4                     |
| 20  | ttcgacgctgnatcatat  | 3 5-hydroxytryptamine (serotonin) receptor 2 (Htr2a) |
| 21  | taaaagctnntagccata  | 2 Fem-1 homolog (C. elegans) (Fem1c)                  |
| 22  | taggcatncagggcga    | 4 Fem-1 homolog (C. elegans) (Fem1c)                  |
| 23  | tcgcctgtnatgcctta   | 1 Fem-1 homolog (C. elegans) (Fem1c)                  |
| 24  | tgcctctggnacccctcg  | 3 GCIP-interacting protein p29                        |
| 25  | egecatgtntaaaacccgg | 2 Zinc finger protein 100 (Zfp100)                    |
| 26  | gtceactctnccaacta   | 4 RIKEN cDNA 9330159N05                               |
| 27  | aagtcatngnagctctc   | 4 RIKEN cDNA 9030407H20                               |
| 28  | tctctcatgtgtagtttt   | 3 RIKEN cDNA 170025D19                                |
| 29  | tggagacttaattgga    | 2 RIKEN cDNA 2410015A15                               |
| 30  | ggccgtgtnaggtggttg   | 2 RIKEN cDNA 4933405K18                               |
| 31  | tcgggttgntaatcttaa   | 1 RIKEN cDNA 6820443006                               |
| 32  | tgcctctntatorctg     | 1 RIKEN cDNA A630095P14                               |
| 33  | gtgcctgntacccccc    | 1 RIKEN cDNA A930024E05                               |
Table 2  Target site sequences of ribozymes and siRNA constructs.

| Rz/siRNA  | Target site sequence                           |
|-----------|-----------------------------------------------|
| Rz 19-1   | ccgctgctgnactccctca                         |
| Rz19-2    | agaacctgcnacccatget                        |
| Rz 19-3   | cctcacagt naccaagaac                        |
| Rz19-4    | gatectctenagctcaac                         |
| Rz19-5    | aagagetgnaeactgaag                           |
| Rz 21-1   | gaccegaaganacacacgcgac                    |
| Rz 21-4   | eetcgctgacagcagcc                           |
| siRNA 19-84 | GttGCtCACTtCAAGAGAG                     |
| siRNA 19-440 | GtGaGaACATCAAGAtATC                  |
| siRNA 21-59 | AGaGtTGatTGAtTCAAC                    |
| siRNA 21-625 | AtAGATTTTCATCATAtTCA               |
| siRNA Fem-1000 | ATTTTCATGtTATAAAGGAC            |
| siRNA Fem-2203 | GtCAtAAAACCTTgCACAgAC          |
Use of a randomized hybrid ribozyme library for identification of genes involved in muscle differentiation

Renu Wadhwa, Tomoko Yaguchi, Kamaljit Kaur, Eigo Suyama, Hiroyuki Kawasaki, Kazunari Taira and Sunil C. Kaul

J. Biol. Chem. published online September 24, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407428200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts