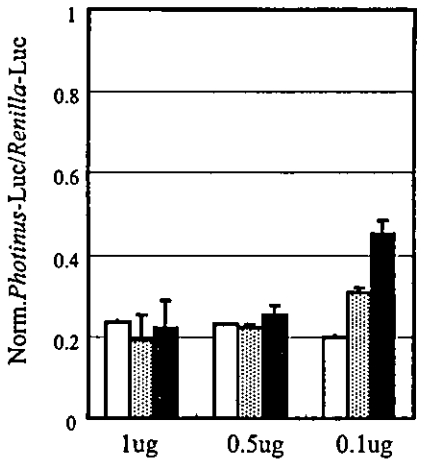

Fig. 7. RNAi induction by short-hairpin RNAs during myogenic differentiation of C2C12 cells. The pRNA-U6.1/Neo/siFluc plasmid (GenScript), which can express a short-hairpin RNA (shRNA) against *Photinus luciferase*, and pRNA-U6.1/Neo empty vector (GenScript) as a control were used. The pGL3-control and phRL-TK plasmids together with a decreasing amount of each of the pRNA-U6.1/Neo/siFluc and pRNA-U6.1/Neo (a negative control) plasmids, from 1 to 0.1 μg, were cotransfected into C2C12 cells. The expression of luciferase was examined 24 h after the transfection. Ratios of normalised target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in Fig. 2. Open, dotted, and solid bars indicate the data in C2C12 cells that differentiated for 0 (undifferentiated), 2, and 7 days, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.

differentiation of C2C12 cell may be caused by losing the stability of functional RISCs in the differentiated C2C12 myotubes.

RNAi induction by short-hairpin RNAs in C2C12 cells

Because Dicer appears to be required for the process of short-hairpin RNAs (shRNAs) into siRNA duplexes, it may be of interest to see if shRNAs can induce RNAi in C2C12 myotubes which barely express *Dicer*. To examine this, we introduced a shRNA expression plasmid against *Photinus* luciferase, pRNA-U6.1/Neo/siRNA, together with the reporter plasmids carrying the *Photinus* and *Renilla* luciferase genes into C2C12 myoblast and myotubes. The results indicate that the shRNA expression plasmid, or shRNAs can induce RNAi in either C2C12 myoblast or myotube (Fig. 7), suggesting that the Dicer protein could be present in those cells. An interesting point to note is that a decrease in the RNAi activity induced by 0.1 μg pRNA-U6.1/Neo/siRNA was observed in C2C12 myotubes that differentiated for 7 days. This may be caused by a possible decrease in the amounts of Dicer and eIF2C1~4 in the cells. To further evaluate the results and a possible relationship between the quantitative level of either Dicer or eIF2C1~4 and RNAi activity, more extensive studies must be conducted.

Integrity of mammalian RNAi

Our previous study has demonstrated that RNAi activity induced by synthetic siRNA duplexes in post mitotic neurons persists for at least 3 weeks, i.e., a long-lasting RNAi activity occurs in mammalian neurons [29]. Our present and previous studies, therefore, suggest that there is a significant difference in the duration of RNAi activity between muscle and neuron, both of which are terminally differentiated and cell cycle-arrested cells. Since neither muscle nor neuron probably undergoes a decrease in the number of functional RISCs by cell division, it may be possible that the stability of functional RISCs could differ between muscle and neuron.

The present observations further suggest the possibility that a little amount of either Dicer or eIF2C1~4 might be sufficient for activation of mammalian RNAi. This seems to be an important point for understanding mammalian RNAi, and further studies on the contribution of either Dicer or eIF2C1~4 to mammalian RNAi must be conducted.

Finally, all the data presented here lead us to the possibility that RNAi may be applicable for a creation of possible model cells and/or model animals for inherited muscular diseases, for example, muscular dystrophy.

Acknowledgments

We thank Drs. Ojima and Takeda (National Institute of Neuroscience) for their technical advice on the preparation of muscle fibers from mouse extensor digitorum longus. This work was supported in part by a Grant-in-Aid from the Japan Society for the Promotion of Science and by research grants from the Ministry of Health, Labor and Welfare in Japan.

References

[1] P.A. Sharp, RNAi and double-strand RNA, Genes Dev. 13 (1999) 139–141.
[2] J.M. Bosher, M. Labouesse, RNA interference: genetic wand and genetic watchdog, Nat. Cell Biol. 2 (2000) E31–E36.
[3] H. Vaucheret, C. Beclin, M. Fagard, Post-transcriptional gene silencing in plants, J. Cell Sci. 114 (2001) 3083–3091.
[4] H. Cerutti, RNA interference: traveling in the cell and gaining functions?, Trends Genet. 19 (2003) 39–46.
[5] S.M. Hammond, E. Bernstein, D. Beach, G.J. Hannon, An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells, Nature 404 (2000) 293–296.
[6] P.D. Zamore, T. Tuschl, P.A. Sharp, D.P. Bartel, RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals, Cell 101 (2000) 25–33.
[7] E. Bernstein, A.A. Caudy, S.M. Hammond, G.J. Hannon, Role for a bidentate ribonuclease in the initiation step of RNA interference, Nature 409 (2001) 363–366.
[8] S.M. Elbashir, W. Lendeckel, T. Tuschl, RNA interference is mediated by 21- and 22-nucleotide RNAs, Genes Dev. 15 (2001) 188–200.

[9] P. Svoboda, P. Stein, H. Hayashi, R.M. Schultz, Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference, Development 127 (2000) 4147–4156.
[10] F. Wianny, M. Zernicka-Goetz, Specific interference with gene function by double-stranded RNA in early mouse development, Nat. Cell Biol. 2 (2000) 70–75.
[11] E. Billy, V. Brondani, H. Zhang, U. Muller, W. Filipowicz, Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines, Proc. Natl. Acad. Sci. USA 98 (2001) 14428–14433.
[12] S. Yang, S. Tutton, E. Pierce, K. Yoon, Specific double-stranded RNA interference in undifferentiated mouse embryonic stem cells, Mol. Cell. Biol. 21 (2001) 7807–7816.
[13] M.R. Player, P.F. Torrence, The 2-5A system: modulation of viral and cellular processes through acceleration of RNA degradation, Pharmacol. Ther. 78 (1998) 55–113.
[14] M. Gale Jr., M.G. Katze, Molecular mechanisms of interferon resistance mediated by viral-directed inhibition of PKR, the interferon-induced protein kinase, Pharmacol. Ther. 78 (1998) 29–46.
[15] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.
[16] S. Matsuda, Y. Ichigotani, T. Okuda, T. Irimura, S. Nakatsugawa, M. Hamaguchi, Molecular cloning and characterization of a novel human gene (HERNA) which encodes a putative RNA-helicase, Biochim. Biophys. Acta 1490 (2000) 163–169.
[17] R.H. Nicholson, A.W. Nicholson, Molecular characterization of a mouse cDNA encoding Dicer, a ribonuclease III ortholog involved in RNA interference, Mamm. Genome 13 (2002) 67–73.
[18] P. Provost, D. Dishart, J. Doucet, D. Frendewey, B. Samuelsson, O. Radmark, Ribonuclease activity and RNA binding of recombinant human Dicer, EMBO J. 21 (2002) 5864–5874.
[19] H. Zhang, F.A. Kolb, V. Brondani, E. Billy, W. Filipowicz, Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP, EMBO J. 21 (2002) 5875–5885.
[20] K.S. Yan, S. Yan, A. Farooq, A. Han, L. Zeng, M.M. Zhou, Structure and conserved RNA binding of the PAZ domain, Nature 426 (2003) 468–474.
[21] J. Martinez, A. Patkaniowska, H. Urlaub, R. Luhrmann, T. Tuschl, Single-stranded antisense siRNAs guide target RNA cleavage in RNAi, Cell 110 (2002) 563–574.
[22] N. Doi, S. Zenno, R. Ueda, H. Ohki-Hamazaki, K. Ui-Tei, K. Saigo, Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors, Curr. Biol. 13 (2003) 41–46.
[23] J.D. Rosenblatt, A.I. Lunt, D.J. Parry, T.A. Partridge, Culturing satellite cells from living single muscle fiber explants, In Vitro Cell. Dev. Biol. Anim. 31 (1995) 773–779.
[24] D. Yaffe, O. Saxel, Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle, Nature 270 (1977) 725–727.
[25] H. Hohjoh, RNA interference (RNAi) induction with various types of synthetic oligonucleotide duplexes in cultured human cells, FEBS Lett. 521 (2002) 195–199.
[26] S.M. Hammond, S. Boettcher, A.A. Caudy, R. Kobayashi, G.J. Hannon, Argonaute2, a link between genetic and biochemical analyses of RNAi, Science 293 (2001) 1146–1150.
[27] R.W. Williams, G.M. Rubin, ARGONAUTE1 is required for efficient RNA interference in *Drosophila* embryos, Proc. Natl. Acad. Sci. USA 99 (2002) 6889–6894.
[28] T. Holen, M. Amarzguioui, M.T. Wiiger, E. Babaie, H. Prydz, Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor, Nucleic Acids Res. 30 (2002) 1757–1766.
[29] K. Omi, K. Tokunaga, H. Hohjoh, Long-lasting RNAi activity in mammalian neurons, FEBS Lett. 558 (2004) 89–95.