Efficient total synthesis of (+)-negamycin, a potential chemotherapeutic agent for genetic diseases†

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Received (in Cambridge, UK) 28th January 2008, Accepted 25th February 2008
First published as an Advance Article on the web 18th March 2008
DOI: 10.1039/b801498a

Herein, we describe an efficient strategy for the total synthesis of (+)-negamycin using commercially available achiral *N*-Boc-2-aminoacetaldehyde as starting material with 42% overall yield for a limited number of steps.

(+)-Negamycin (**1**, Scheme 1), an unusual antibiotic containing a hydrazine peptide bond was isolated for the first time by Umezawa *et al.* in 1970 from culture filtrates of three strains related to *Streptomyces purperfuscus*. This natural product exhibits very low acute toxicity and strong inhibitory activity against multiple drug-resistant enteric Gram-negative bacteria including *Pseudomonas aerginosa*.[1] (+)-**1**'s anti-microbial activity is derived from a genetic miscoding on bacterial ribosomal systems, and thereby leading to a specific inhibition of protein biosynthesis.[2] Because this miscoding causes read-through of termination signals, considerable attention is focused on (+)-**1** as a potential therapeutic agent against genetic diseases. Indeed, the aminoglycoside antibiotic gentamicin and the less toxic negamycin both restore dystrophin expression in skeletal and cardiac muscles of mdx mice, an animal model of Duchenne muscular dystrophy (DMD) with a nonsense mutation in the dystrophin gene.[3] Therefore, an efficient shortened synthetic route of (+)-**1** and its derivatives appears significant to develop promising new therapeutic candidates for DMD and other diseases caused by nonsense mutations. The first total synthesis of (+)-**1** from D-galacturonic acid was reported in 1972 and confirmed the assigned structure of the natural product.[4] Over three decades, numerous total syntheses have been reported on both racemic and optically active (+)-**1** but with moderate overall yield.[5]

Our fast and efficient route consists first on an asymmetric allylboration of *N*-Boc-glycinal **5** using the established Brown's procedure for preparation of chiral allylic alcohols[6] that led to a corresponding chiral intermediate (Scheme 1). This resulting chiral amino alcohol was directly engaged without further purification to form the target oxazolidine **4** by treatment with 2,2-dimethoxypropane (DMP) in the presence of boron trifluoride diethyl ether complex ($BF_3{\cdot}Et_2O$) in acetone. As a result, **4** was generated in high yield after purification by silica gel column chromatography (90%). To prepare the key intermediate **3**, a cross-metathesis (CM) reaction between **4** and *tert*-butyl acrylate **6** was investigated. Although the efficiency of ruthenium-based catalysts for ring-opening metathesis polymerization (ROMP) and ring-closing metathesis (RCM) is now well established, most alkene CM variants have fewer successful applications because of the multiple possible side reactions that cause relatively low synthetic yields.[7] Because our substrates are categorized as rapid and slow homodimerizable compounds according to Grubbs *et al.*'s empirical model for predicting the outcome of CM reactions,[8] we screened different reaction conditions to avoid forming unwanted dimers and selectively provide the target compound **3** by varying catalysts (Grubbs first [Ru-**I**] and second-generation [Ru-**II**] catalysts), amount of reactant (1 or 5 equiv. of **6**), duration of reaction as well as heating method (conventional or microwave-assisted heating).

As detailed in the ESI,† the conversion and chemoselectivity enhancements are definitely more pronounced for [Ru]-**II** than [Ru]-**I**. Furthermore, we observed that microwave irradiation drastically shortened the CM reaction time by 20-fold. As it pertains to microwave-assisted synthesis, this acceleration is commonly attributed to the very high local temperatures and the ease to which microwave irradiation reaches such conditions but the scientific community is still divided in opinion on the involvement of a specific non-thermal effect induced by the dielectric heating produced using microwaves.[9] Interestingly, although our observations provide a new example for the thermal effect, the involvement of such "specific effect" is neither confirmed nor disproved in these reaction conditions. Bargiggia *et al.* arrived to similar conclusions while studying CM reactions[10] and Garbaccia *et al.* have described similar observations for RCM reactions in 2003.[11] As a result, the desired product **3** was isolated with 83% yield. NOE experiments revealed that the stereochemistry of the olefin moiety in **3** was an *E* configuration ($J_{\text{vinylic protons}}$ = 15.7 Hz). Thus, the desired chiral intermediate **3** was obtained with 75% yield after two steps from achiral *N*-Boc-glycinal **5**.

With intermediate **3** in hand, our focus shifted toward the asymmetric Michael addition reaction. Recently, Node *et al.*

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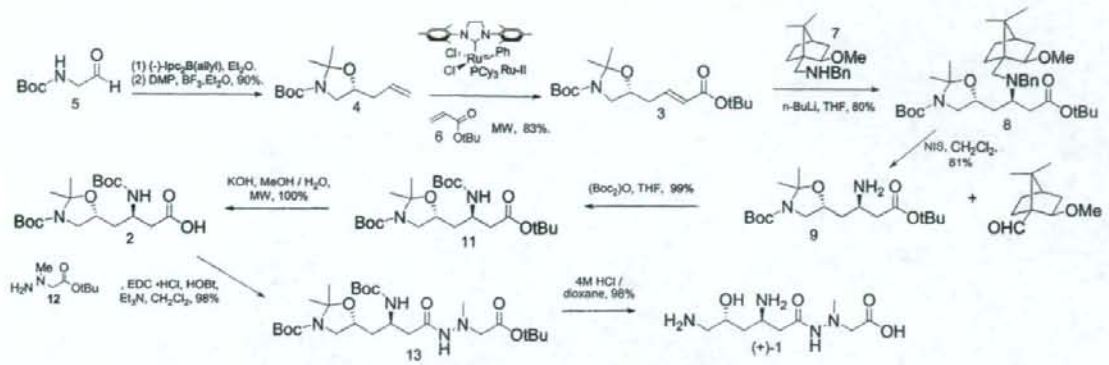
† Electronic supplementary information (ESI) available: Analytical data. See DOI: 10.1039/b801498a



Scheme 1 Total synthesis of (+)-negamycin.

reported a highly stereoselective asymmetric Michael addition toward *tert*-butyl α,β-unsaturated carbonyl compounds using chiral amine **7**.[12] This approach, when applied to the α,β-unsaturated *tert*-butyl ester **3**, allowed the introduction of the amine moiety with an excellent enantiomeric excess.[13] The chiral reagent **7** was prepared from the corresponding ketopinic acid[12] and reacted with **3** in the presence of *n*-BuLi in THF at −78 °C to afford compound **8** as a single diastereomer (de >99%) in 80% yield after purification. Removal of both benzyl and 2-methoxybornyl protecting groups located on the same amine moiety could be achieved efficiently using 4 equiv. of *N*-iodosuccinimide (NIS) in dichloromethane to obtain free amino compound **9** in 81% yield. This deprotection proceeded by oxidation with NIS to imine and subsequent spontaneous hydrolysis to afford *tert*-butyl esters of β-amino acids and 2-methoxy-D-bornylaldehyde.[12] No epimerization was observed during this reaction. Furthermore, one of the advantages of the protocol is that the initial chiral inducer **7** can be easily regenerated from 2-methoxy-D-bornaldehyde, generated during the cleavage by reductive amination, using benzylamine in the presence of sodium cyanoborohydride (data not shown).

The last part of the synthesis of (+)-**1** consisted of introducing a hydrazine unit, prior to a final deprotection. A Boc-protection of **9** using standard procedures was first quantitatively performed to afford *N*-protected *tert*-butyl ester **11**, that was then efficiently converted to acid **2** by a microwave-assisted saponification with 2M KOH in MeOH, and coupling with hydrazine unit **12** was then performed using the classical EDC·HCl–HOBt method. The synthesis of hydrazine **12** was achieved by reacting *N*-methyl hydrazine with *tert*-butyl bromoacetate with 40% yield after purification. Deprotection of compound **13** and purification by ion exchange chromatography on Amberlite CG50 (NH_4^+ form) afforded the target compound (+)-**1** in 98% yield, $[\alpha]_D^{25.2}$ +2.4° (*c* 0.36, H_2O), lit. $[\alpha]_D^{29.0}$ + 2.5° (*c* 2.00, H_2O). The final compound was fully characterized and compared with the published data for the natural product to confirm the success of this new total synthesis of (+)-**1** (*e.g.* ^{1}H NMR data for natural and synthesized (+)-**1**, available in ESI†). Furthermore, the *in vivo* read-through activity of termination codons during protein biosynthesis[3] of the synthesized (+)-**1** in mice was very similar to that of the native (+)-**1** (data not shown). Further derivatization of the **1** structure using the above synthetic methodology will contribute to a better understanding of the structure–activity relationship of **1** and the development of more potent compounds with efficient read-through activity. Studies in this regard are currently in progress and details pertaining to the biological activity will soon be published elsewhere.

In conclusion, the proposed synthetic route for the total synthesis of optically active (+)-negamycin starting from *N*-Boc-glycinal **5**, led to the desired product with a total yield of 42% over only eight steps. To our knowledge, this study represents the most efficient strategy to prepare (+)-**1**. Current efforts with this new synthetic approach are now expanding into medicinal chemistry to discover new drug candidates with potent read-through activity for Duchenne muscular dystrophy. The chemical biology of negamycin is also now being investigated to better understand its read-through mechanism.

This research was supported by the Research Grant (17A-10) for Nervous and Mental Disorders from the Ministry of Health, Labour and Welfare. T. R. is grateful for the Postdoctoral Fellowship of JSPS. We thank Dr J.-T. Nguyen for valuable help during manuscript preparation.

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ZOOLOGICAL SCIENCE 25: 1066–1074 (2008)

The Expression of Myogenin, but Not of MyoD, is Temperature-Sensitive in Mouse Skeletal Muscle Cells

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Homothermal animals need to keep their body temperature within a narrow range. Only a few degrees Celsius change in temperature has a dynamic influence on many physiological processes. To investigate the effect of the body temperature on muscle cell differentiation, we cultured the mouse myoblast cell lines C2C12 and Sol8 at lower temperatures than mouse body temperature. At 38°C, the cells fused into multinucleated myotubes within 4 days after the induction of differentiation. However, myotube formation was blocked at 30°C, whereas it was delayed but relatively normal at 35°C. The myoblasts expressed MyoD, but not myogenin, at 30°C. Id3, which acts as a negative regulator of myogenic regulatory factors (MRFs), was expressed at a higher level at 30°C than at 38°C, whereas the expression level of E2A, which acts as a positive regulator of MRF expression, exhibited no difference between these temperatures. We also found that the expression of muscle-enriched microRNAs decreased at 30°C. In addition, we investigated the expressions of MyoD and myogenin during mouse satellite-cell activation in single-fiber culture as an in-vivo model, and found that the expression of myogenin, but not of MyoD, was inhibited. These results suggest that skeletal muscle formation can be regulated by temperature, and that the physiological body temperature plays a crucial role in the myogenesis of homothermal animals.

Key words: myogenesis, MyoD, myogenin, temperature, differentiation, skeletal muscle, transcriptional regulation, mouse

INTRODUCTION

The emergence of homothermal animals is one of the most significant events in vertebrate evolution (Colbert, 1980). Homothermal animals are able to keep their body temperatures constant in spite of variation in environmental temperatures. Body temperature has recently been shown to play an important role in various physiological processes in mammals, including insulin secretion (Togashi et al., 2006) and hippocampal neural activity (Shibasaki et al., 2007). However, the effect of body temperature on cell differentiation has not yet been sufficiently elucidated. We investigated the effect of temperature on myogenic differentiation, of which the molecular cascade is relatively well understood compared with other cell types.

The myogenic regulatory factors (MRFs) MyoD, myf-5, myogenin, and MRF4 are a family of basic helix-loop-helix (bHLH) transcriptional factors responsible for the induction and maintenance of muscle differentiation (Berkes and Tapscott, 2005). MyoD and myf-5 are required for the initial determination of a myogenic lineage, whereas myogenin and MRF4 are responsible for the commitment to myogenesis. MyoD or myf-5 single-knockout mice show no morphological abnormalities in the skeletal muscles (Braun et al., 1992; Rudnicki et al., 1992), but MyoD/myf-5 double-knockout mice fail to generate myoblasts (Rudnicki et al., 1993). That is, MyoD and myf-5 have overlapping, or redundant, functions (Braun and Arnold, 1994). In myogenin-knockout mice, myoblasts are formed in the correct place but do not fuse into mature myofibers (Hasty et al., 1993; Nabeshima et al., 1993).

The expressions of MRFs are regulated by various chemical environmental factors, such as growth factors and extracellular matrices. For instance, IGF-I induces the elevation of myogenin expression and stimulates myogenic differentiation (Florini et al., 1996). The inhibition of collagen synthesis prevents muscle cell differentiation (de la Haba and Bricker, 1981) by blocking the expression of MyoD and myogenin (Saitoh et al., 1992). In addition, some physical environmental factors, such as mechanical and electrical stimulation, were shown to influence skeletal muscle growth and/or differentiation in vivo (Goldspink et al., 1991) and in vitro (Vandenburgh et al., 1991). Heat stress was also shown to induce muscular hypertrophy due to an increase in heat-shock protein (HSP) 72 (Uehara et al., 2004).

In this study, we cultured mouse myoblast cell line C2C12, which is a well-established model for studying myogenesis in vitro, at lower temperature than the mouse body temperature (38°C). We report here that mouse myoblast cells cultured at 30°C did not undergo terminal differentiation due to the inhibition of myogenin expression.

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doi:10.2108/zsj.25.1066

MATERIALS AND METHODS

Cell culture

A C2C12 cell line (Blau et al., 1983), a subclone of the C2 cell line isolated from the thigh muscle of an adult C3H mouse (Yaffe and Saxel, 1977), was kindly provided by Dr. Yoichi Nabeshima (Kyoto University, Kyoto, Japan). A Sol8 cell line (Daubas et al., 1988) was kindly provided by Dr. Muthu Periasamy (Ohio State University, OH, USA). The cells were grown in DMEM (high-glucose) (Invitrogen, Carlsbad, CA, USA) containing 20% FBS (SAFC Biosciences, Lenexa, KS, USA), 50 U/ml penicillin, and 50 mg/ml streptomycin in plastic dishes (AGC Techno Glass, Chiba, Japan) coated with 1% bovine skin gelatin (Sigma-Aldrich, St. Louis, MO, USA) at 38°C in 5% CO_2. At a point near confluence, the medium was replaced with MEM (Invitrogen) containing 10% horse serum (Invitrogen), 50 U/ml penicillin, and 50 mg/ml streptomycin to induce differentiation, and the cells were then cultured at 30°C, 35°C, or 38°C in 5% CO_2 for 4 days (C2C12 cells) or 3 days (Sol8 cells). The medium was changed every other day.

Single-fiber culture

Single muscle fibers were prepared as described previously (Nagata et al., 2006). Briefly, the extensor digitorum longus (EDL) muscles were removed from adult C3H/HeNJc1 mice (CLEA Japan, Tokyo, Japan) by excising the tendons, and treated with 0.1% collagenase Type I (Sigma-Aldrich) in DMEM at 38°C for 2 h. Single fibers were liberated from the EDL muscles by gentle pipetting and cultured in DMEM (high-glucose) containing 20% FBS, 50 U/ml penicillin and 50 mg/ml streptomycin. Experimental fibers were cultured at 38°C for 24 h and then at 30°C for 2 days, while the control fibers were cultured at 38°C for 3 days. Animal experimentation was carried out according to the guidelines of the animal committee of The University of Tokyo.

Immunofluorescence

Cells or mouse muscle fibers were fixed with 10% formalin in PBS for 20 min at room temperature, followed by treatment with 100% methanol for 15 min at room temperature. Non-specific protein binding was blocked by incubation with 10% HS, 3% BSA in PBS. The samples were incubated with primary antibodies [mouse anti-myosin heavy chain, MF20 (1:2; Developmental Studies Hybridoma Bank, Iowa City, LA, USA); mouse anti-MyoD (1:50; Dako Cytomation, Glostrup, Denmark); rabbit anti-MyoD (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-myogenin, F5D (1:1; a gift from Dr. Woodring E. Wright of the University of Texas, Southwestern Medical Center, TX, USA); and rabbit anti-E2A.E12 (1:50; Santa Cruz Biotechnology)] for 1 h at room temperature, followed by Alexa Fluor dye-conjugated secondary antibodies (1:400; Invitrogen) for 1 h at room temperature. The antibodies were diluted with 3% BSA in PBS. The cell nuclei were stained with Hoechst 33258 (Sigma-Aldrich). The samples were observed by using a fluorescence microscope (Axioplan, Carl Zeiss, Oberkochen, Germany), and images were taken with a digital camera system (VB-7000, Keyence, Osaka, Japan).

mRNA isolation and RT-PCR

Four days after the induction of differentiation (day 4), total RNA was isolated from the C2C12 cells using RNAZol B reagent (Tel-Test, Friendswood, TX, USA) in accordance with the manufacturer's protocol. Reverse transcription of the isolated mRNA and amplification of cDNA were performed with 100 ng RNA by using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen). PCR conditions were 94°C for 15 s, 60°C for 30 s, and 55°C for 45 s for 23 cycles (Ids, E2A) or 25 cycles (MyoD, myogenin). Primer sequences for MyoD, myogenin, E2A, Id1, Id2, Id3, and β-actin were as follows: MyoD (Shi et al., 2005), 5'-AGG CTC TGC TGC GCG ACC-3' and 5'-TGC AGT CGA TCT CTC AAA GCA CC-3'; myogenin (Kadota et al., 2000), 5'-GAG CTG TAT GAG ACA TCC CC-3' and 5'-GTA AGG GAG TGC AGA TTG TG-3'; E2A (Sun et al., 2007), 5'-TAC CCC TCC GCC AAG ACC-3' and 5'-TTG GGG GAT AAG GCA CTG-3'; Id1 (Sakuma et al., 2005), 5'-GTC CTG CTC TAC GAC ATG AAC G-3' and 5'-GAT CAA ACC CTC TAC CCA CTG G-3'; Id2 (Saika et al., 2006), 5'-AAA ACA GCC TGT CGG ACC AC-3' and 5'-CTG GGC ACC AGT TCC TTG AG-3'; Id3 (Riechmann et al., 1994), 5'-AAG GCG CTG AGC CCG GTG C-3' and 5'-TCG GGA GGT GCC AGG ACG-3'; β-actin (Riechmann et al., 1994; Shi et al., 2005), 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3' and 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3' (349 bp), or 5'-AGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' and 5'-CTA GAA GCA CTT GCG GTG CAC GAT GGA AGG G-3' (661 bp). PCR products were resolved by electrophoresis on 2% agarose gels and stained with 1 μM SYTO60 Red Fluorescent Nucleic Acid Stain (Invitrogen) for 20 min. The band intensity was measured by using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and normalized to the β-actin band intensity at each temperature.

MicroRNA isolation and RT-PCR

Small RNAs were isolated from the C2C12 cells on day 4 by using a *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX, USA) in accordance with the manufacturer's protocol. Reverse transcription of the isolated small RNAs and amplification of cDNA were performed with 10 ng RNA by using a *mir*Vana qRT-PCR miRNA Detection Kit (Ambion) and *mir*Vana qRT-PCR Primer Sets (Ambion). The primers for microRNA(miR)-1, miR-133a, miR-181a, miR-206, and 5S rRNA were hsa-miR-1, hsa-miR-133a, hsa-miR-181a, hsa-miR-206, and 5s, respectively. PCR was performed for 20 cycles. The PCR products were resolved by electrophoresis on 3.5% agarose gels and stained with 1 μM SYTO60 dye for 20 min. The band intensity was normalized to the 5S rRNA band intensity at each temperature.

Statistical analysis

The cell nuclei expressing MyoD or myogenin in the C2C12 cells or Sol8 cells were counted in more than three different views for each sample, and the ratio of MyoD- or myogenin-positive nuclei to total nuclei (more than 150 nuclei per view) was calculated. For the single-fiber culture analysis, the satellite cells of 51 myofibers (38°C) or 31 myofibers (30°C) were counted and the ratios of myogenin-positive satellite cells to MyoD-positive satellite cells per myofiber were calculated. The ratios are expressed as mean percentages (%) ±SD. The *t*-test was used to evaluate the significance of differences between ratios.

RESULTS

Myotube formation in C2C12 myoblasts is retarded at 35°C and blocked at 30°C

To investigate the effect of low culture temperature on the differentiation of mouse myoblasts, C2C12 cells were grown in 20% FBS/DMEM at 38°C and differentiated in 10% horse serum/MEM at 35°C or at 30°C. As a control, C2C12 cells were differentiated at 38°C. The C2C12 myoblasts in the control cultures fused into multinucleated myotubes within 4 days after the induction of differentiation (day 4), whereas the myoblasts cultured at 35°C formed myotubes on day 5. Myotube formation was delayed for a day at 35°C, but the myotubes showed no significant changes in appearance. At 30°C, however, no myotubes were observed even when the myoblasts were cultured in differentiation medium for 10 days. When the myoblasts were returned to 38°C after being cultured at 30°C for 4 days, they formed myotubes again within 4 days. That is, the myogenic

differentiation of the C2C12 cells was reversibly blocked at 30°C.

To examine muscle-specific protein synthesis in the C2C12 cells cultured at 30°C, the cells were stained on day 4 with monoclonal antibody against sarcomeric myosin heavy chain (MyHC), MF20 (Bader et al., 1982). Immunofluorescence staining revealed that the myoblasts did not express MyHC at all at 30°C, whereas many large multinucleated myotubes were formed and expressed MyHC at 38°C (Fig. 1).

To determine whether apoptosis was induced by the change in temperature from 38°C to 30°C, we performed a TUNEL assay using C2C12 cells 6 h, 12 h, 24 h or 4 days after the induction of differentiation. No significant apoptosis occurred at any time point of the analysis (data not shown).

C2C12 cells express MyoD, but not myogenin, at 30°C

C2C12 cells were fixed on day 4 and immunostained for MyoD, which is required for myogenic determination, and myogenin, which mediates terminal differentiation. The C2C12 cells in the control cultures expressed both MyoD and myogenin. However, myogenin expression at 30°C was significantly decreased compared with the expression at 38°C, while there was no significant difference in MyoD expression between the two conditions (Fig. 2). The nuclei appeared to be spread out and flattened at 30°C, for a reason not yet elucidated.

Sol8 cells also do not express myogenin at 30°C

To demonstrate that the inhibition of myogenesis at 30°C occurred not only in the C2C12 cell line but also occurs in mouse skeletal muscle cells generally, we also cultured Sol8 cells, which were derived from primary cultures of the soleus muscle isolated from a C3H mouse (Daubas et al., 1988). Sol8 cells were grown in 20% FBS/DMEM at 38°C and differentiated in 10% horse serum/MEM at 30°C or at 38°C in the same way as the C2C12 cells. The Sol8 cells fused into multinucleated myotubes within 3 days after the induction of differentiation, which was earlier than the C2C12 cells. Sol8 cells were fixed on day 3 and immunostained for MyoD and myogenin. The Sol8 cells cultured at 30°C showed the same immunostaining patterns as the C2C12 cells cultured at 30°C; that is, they expressed MyoD, but not myogenin (Fig. 3).

Myogenin expression is inhibited at the transcriptional level at 30°C

To determine whether myogenin expression was inhibited at the transcriptional level at 30°C, total RNA was isolated on day 4 from C2C12 cells cultured at 30°C or at 38°C, and RT-PCR for MyoD and myogenin was performed. The myogenin band intensity at 30°C was less than 15% of that at 38°C (Fig. 4), which showed that the inhibition of myogenin expression in C2C12 cells cultured at 30°C occurred at the transcriptional level.

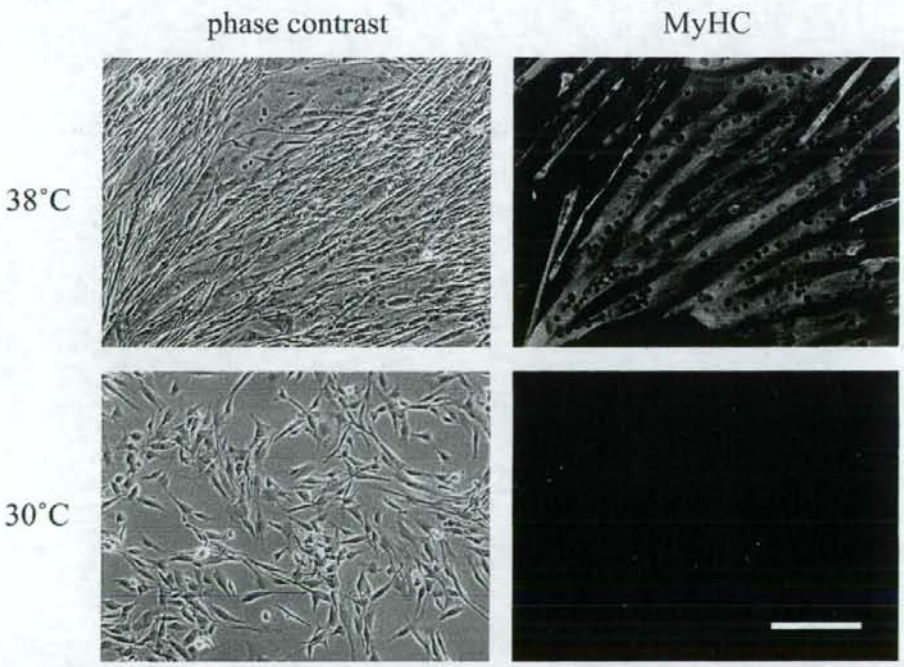

Fig. 1. MyHC expression in C2C12 cells cultured at 30°C or at 38°C. The cells were cultured in differentiation medium at 30°C or at 38°C for 4 days and immunostained with MF20, an anti-MyHC antibody. Many large multinucleated myotubes expressing MyHC were formed at 38°C. The myoblasts did not express MyHC, and myoblast fusion was blocked, at 30°C, suggesting that myogenic differentiation was inhibited at 30°C. Scale bar, 200 μm.

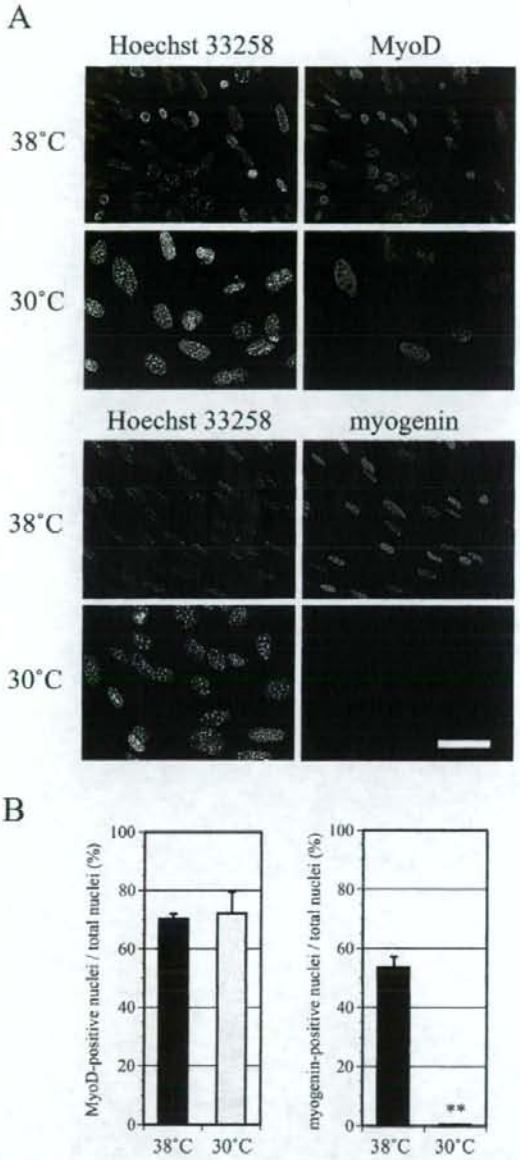

Fig. 2. Immunofluorescence analysis of MyoD and myogenin in C2C12 cells cultured at 30°C or at 38°C. **(A)** Cells were fixed on day 4 and immunostained with anti-MyoD and anti-myogenin antibodies, and also stained with Hoechst 33258. Scale bar, 50 μm. **(B)** Ratios of MyoD- or myogenin-positive nuclei to total nuclei are given as mean percentages±SD. Myogenin expression was significantly lower at 30°C than at 38°C (**p<0.01; *t*-test), whereas there was no significant difference in MyoD expression.

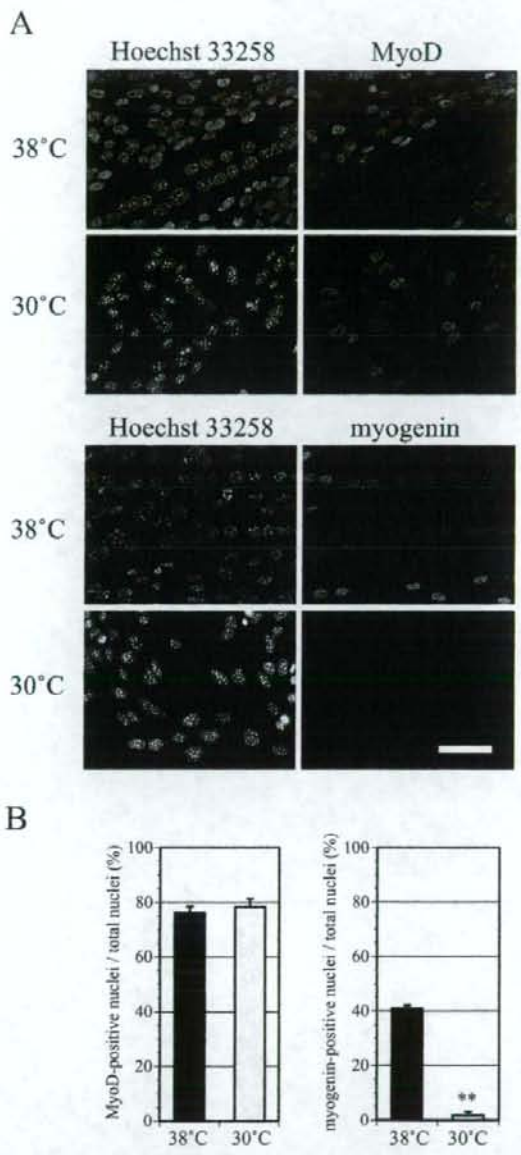

Fig. 3. Expression of MyoD and myogenin in Sol8 cells cultured at 30°C or at 38°C. **(A)** Cells were fixed on day 3 and immunostained with anti-MyoD and anti-myogenin antibodies, and also stained with Hoechst 33258. Scale bar, 50 μm. **(B)** Ratios of MyoD- or myogenin-positive nuclei to total nuclei are given as mean percentages±SD. There was a significant difference in myogenin expression between the cells cultured at 38°C and those cultured at 30°C (**p<0.01; *t*-test), but not in MyoD expression, as was observed in C2C12 cells (Fig. 2).

The E2A expression level does not change at 30°C

We investigated the expression of other transcriptional factors that interact with MRFs as positive or negative regulators, because it was quite possible that MyoD did not function as a transcriptional activator at 30°C. First, we examined the expression of the E2A gene product, which includes E12 and E47 as splice variants (Sun and Baltimore, 1991). E2A is a ubiquitous basic helix-loop-helix (bHLH)

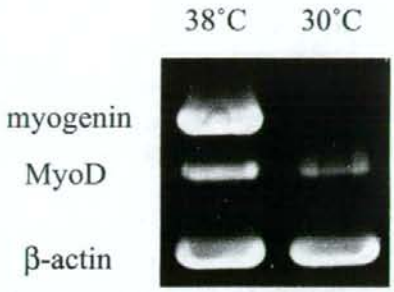

Fig. 4. RT-PCR of MyoD and myogenin in C2C12 cells. Total RNA was isolated from C2C12 cells cultured at 30°C or at 38°C on day 4. The gel was stained with SYTO60 dye and scanned with an infrared imaging system. The expression level of β-actin mRNA, which was almost the same in the cells cultured at 30°C and at 38°C, was used as a positive control. At 30°C, myogenin mRNA expression was extremely low, whereas MyoD mRNA was strongly expressed.

protein forming a heterodimer with MRFs. The MRF/E2A heterodimer binds to a consensus DNA sequence (CANNTG) called an E-box, which is present in promoters or enhancers of muscle-specific genes (Lassar et al., 1989) and activates the transcription of its target genes. We examined by RT-PCR E2A expression in the C2C12 cells cultured at 30°C for 4 days. As controls, cells which were cultured in the growth medium and in which differentiation was not induced (day 0) and cells differentiated at 38°C for 4 days were examined. As shown in Fig. 5A, E2A mRNA expression was low in all samples. The level was the same in myoblasts at day 0 and myotubes differentiated at 38°C, which coincided with the results of a previous study (Sun et al., 2007). Additionally, that previous study showed that the E2A protein level was regulated post-translationally and that its localization changed from the nuclei to both the nuclei and the cytoplasm in C2C12 cells as differentiation progressed from myoblasts to myotubes (Sun et al., 2007). Therefore, we also performed an immunofluorescence

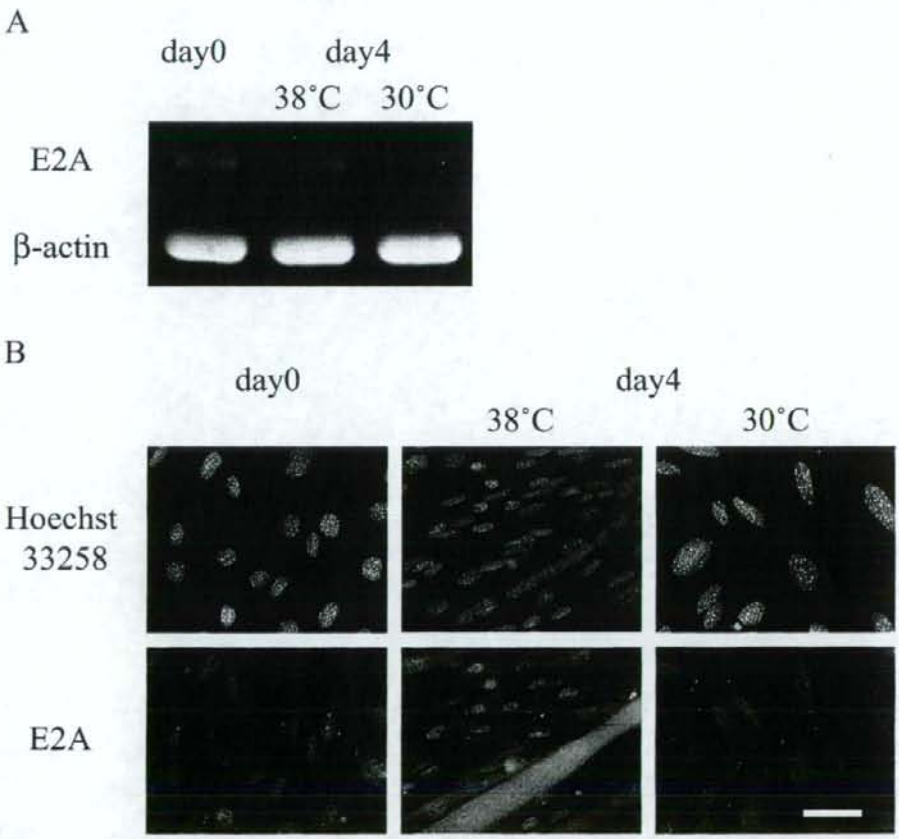

Fig. 5. E2A expression in C2C12 cells. **(A)** Total RNA was isolated from cells before the induction of differentiation (day 0) and from cells cultured for 4 days at 30°C or at 38°C, and RT-PCR was performed. E2A mRNA expression was low in all samples, and its level remained unchanged before and after the induction of differentiation. **(B)** Cells were fixed on day 0 or day 4 and immunostained with anti-E2A antibody, and also stained with Hoechst 33258. E2A proteins were expressed in the nuclei of the myoblasts on day 0 and in both the nuclei and the cytoplasm of the cells differentiated at 38°C. In the cells cultured at 30°C, E2A proteins were also expressed and localized in the nuclei. Scale bar, 50 μm.

analysis to investigate E2A protein expression and its localization. We confirmed that E2A proteins were expressed in the nuclei of myoblasts at day 0 and in both the nuclei and the cytoplasm of cells differentiated at 38°C. In cells cultured at 30°C, E2A proteins were also expressed and localized in the nuclei (Fig. 5B), suggesting that the expression pattern of E2A was not influenced by the low temperature.

Id3 is expressed at higher levels and accumulates in the nuclei at 30°C

We investigated the expression of Ids as negative regulators of MRFs. Ids are a family of dominant negative HLH proteins that contain HLH dimerization domains but lack DNA-binding basic regions (Benezra et al., 1990). Ids disturb MRF/E2A heterodimer formation by binding to E2A with high affinity (Berkes and Tapscott, 2005). The Id family includes Id1, Id2, Id3, and Id4, in which Id1, Id2, and Id3 have been shown to negatively regulate myogenesis (Jen et al., 1992; Melnikova and Christy, 1996; Melnikova et al., 1999). We examined by RT-PCR the expression of Id1, Id2, and Id3 in C2C12 cells, isolating total RNA from the cells before the induction of differentiation (day 0) and from cells differentiated at 30°C or 38°C for 4 days. As shown in Fig. 6, the cells on day 0 expressed all Ids at the highest level, which coincided with the results of a previous study showing that Ids were highly expressed in myoblasts, but decreased upon the initiation of differentiation (Benezra et al., 1990). Compared to the cells differentiated at 38°C, the cells cultured at 30°C expressed Id3 mRNA at higher levels, while the Id1 mRNA expression level was lower. The Id3 band intensity at 30°C was 1.4 times that at 38°C. Id2 mRNA expression was low at both 30°C and 38°C. These results suggest that Id3 might prevent MRFs from heterodimerizing with E2A and thus inhibit myogenic differentiation at 30°C.

MicroRNA expression decreases at 30°C

We investigated the microRNA (miRNA) expression patterns in C2C12 cells cultured at 30°C as one of the indexes of myogenic differentiation. MiRNAs are a class of small non-coding RNAs that negatively regulate gene expression and are involved in many biological processes, such as cell proliferation, differentiation, and apoptosis (Bartel, 2004). It has been shown that several miRNAs are specifically expressed or highly enriched in skeletal muscles and regulate myogenesis (Callis et al., 2007). We examined the expressions of miRNA-1, -133a, -181a, and -206 by RT-PCR, isolating small RNAs from C2C12 cells cultured for 4 days at 30°C or at 38°C. We found that miR-1, -133a, and -206 were regularly expressed at 38°C, as shown in a previous study (Kim et al., 2006), whereas they were hardly detected at 30°C; miR-181a was slightly expressed even at 30°C, but the expression level was lower than that at 38°C (Fig. 7).

Myogenin expression in activated satellite cells is also inhibited at 30°C

We investigated the expressions of MyoD and myogenin during satellite-cell activation as an in-vivo model. Satellite cells are myogenic stem cells (Mauro, 1961) that are mitotically quiescent in adult muscles (Schultz et al., 1978), but can be activated by signals released from crushed myofibers (Chargé and Rudnicki, 2004). The activated satellite cells enter the cell cycle and produce myogenic precursor cells, which differentiate into multinucleated myotubes (Snow, 1977) and then contribute to muscle repair (Zammit and Beauchamp, 2001). We isolated myofibers from C3H mice and incubated them in a medium containing serum which activates satellite cells (Yablonka-Reuveni and Rivera, 1994). After incubating the cells for 24 h at 38°C, we performed an immunofluorescence analysis and found that the satellite cells expressed MyoD but not yet myogenin. The cells were then cultured at 30°C for 2 more days. On day 3, the satellite cells cultured at 38°C for 3 days as a control expressed both MyoD and myogenin, whereas the cells cultured at 30°C for the last 2 days expressed MyoD, but not myogenin (Fig. 8).

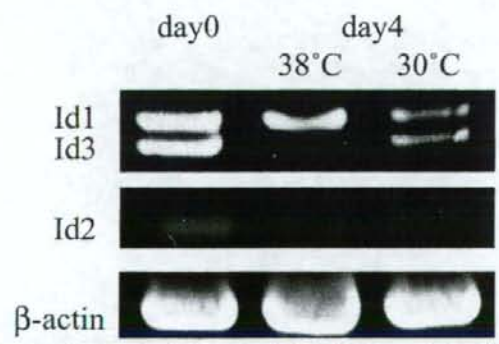

Fig. 6. Id expression in C2C12 cells. Total RNA was isolated from cells before the induction of differentiation (day 0) and from cells cultured for 4 days at 30°C or at 38°C, and RT-PCR was performed for Id1, Id2, and Id3. All Ids were expressed at the highest level on day 0, and their expression levels decreased on day 4 when the cells were cultured at 38°C, but cells cultured at 30°C still expressed Id3 mRNA at higher levels than at 38°C.

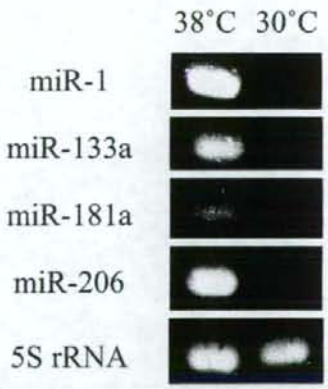

Fig. 7. MicroRNA (miR) expression in C2C12 cells. Small RNAs were isolated on day 4 from C2C12 cells cultured at 30°C or 38°C, and RT-PCR was performed for miR-1, -133a, -181a, and -206. As a positive control, 5S rRNA expression was examined. MiR-1, -133a, and -206 were regularly expressed at 38°C, but were hardly detected at 30°C. MiR-181a was expressed at both 30°C and 38°C, but was decreased in cells cultured at 30°C.

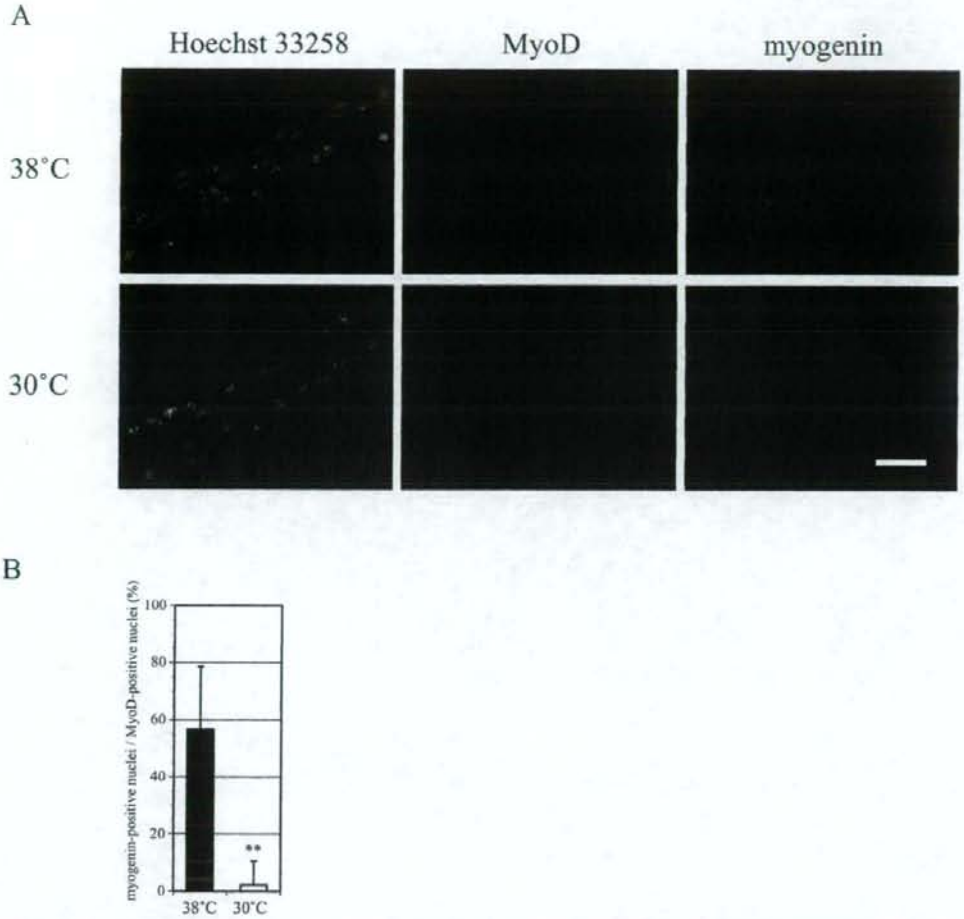

Fig. 8. MyoD and myogenin expression in the activated satellite cells of mouse myofibers. Single muscle fibers were isolated from the EDL muscles of C3H mice and incubated in 20% FBS/DMEM to activate the satellite cells. After 24 h of incubation at 38°C, the myofibers were cultured at 30°C for 2 more days. As a control, the fibers were cultured at 38°C for 3 days. **(A)** Myofibers were fixed and immunostained with anti-MyoD and anti-myogenin antibodies, and also stained with Hoechst 33258. Scale bar, 50 μm. **(B)** Ratios of myogenin-positive nuclei to MyoD-positive nuclei are expressed as mean percentages±SD. Satellite cells cultured at 38°C expressed both MyoD and myogenin, whereas cells cultured at 30°C for the last 2 days expressed MyoD, but not myogenin. There was a significant difference between the ratios (**$p<0.01$; *t*-test).

These results indicate that the inhibition of myogenin expression at 30°C occurs not only in muscle cell lines such as C2C12 and Sol8, but also in the satellite cells of mouse myofibers.

DISCUSSION

To elucidate the inhibitory mechanism of myogenin expression at 30°C, we investigated the expression of E2A and Ids. Myogenin expression is directly activated by MyoD (Hollenberg et al., 1993). So, why was myogenin not expressed at 30°C, even though MyoD was expressed and localized in the nuclei? It might be because MyoD did not attain its transcriptional activation potential at 30°C. E2A proteins, which form a heterodimer with MRFs and act as positive regulators of MRF expression, were expressed at 30°C as well as at 38°C. However, Id3, which disturbs MRF/E2A heterodimer formation and acts as a negative regulator of myogenesis, was expressed at higher levels at 30°C than at 38°C. This suggests that Id3 might prevent MyoD from attaining its transcriptional activation potential at 30°C.

We also investigated by RT-PCR the miRNA expression patterns as indices of myogenic differentiation. In C2C12 cells cultured at 30°C, miR-1, -133a, and -206 were hardly detected, but miR-181a was slightly expressed. MiR-1, -133, and -206 have been shown to have upstream MyoD- and myogenin-binding sites and, therefore, are likely to be regulated by MyoD and/or myogenin (Rao et al., 2006; Rosenberg et al., 2006). On the other hand, miR-181 is expressed upstream of MyoD and induces MyoD expression (Naguibneva et al., 2006). Thus, these results, in which an miRNA acting upstream of MyoD was expressed, but miRNAs acting downstream of MyoD were not, also suggest that

MyoD might not attain its transcriptional activation potential at 30°C.

Maltby et al. (2004) found that myogenin expression in turkey embryos incubated at 35.5°C for 5 to 8 embryonic days was lower than in control embryos incubated at 37.5°C, and that the peak of myogenin expression appeared later in temperature-manipulated embryos. Together with our data, these findings imply that myogenin expression is inhibited at low temperature in birds and mammals.

While only a few studies on the effect of temperature on myogenesis have been carried out in homothermal animals, many studies have been performed using fish, which can adapt to a wide range of water temperatures. Whether or not the expression patterns of MRFs change depending on the water temperature has also been examined in the embryos of many different fish species. No changes have been observed in any developmental stage in the herring (Temple et al., 2001), Atlantic cod (Hall et al., 2003), carp (Cole et al., 2004), and Atlantic halibut (Galloway et al., 2006). On the other hand, in the Atlantic salmon, the expression of myf-5 and MRF4, but not of MyoD and myogenin, is retarded with respect to the somite stage at 2°C compared to that at 8°C (Macqueen et al., 2007). In the rainbow trout, the myogenin expression is delayed but prolonged at 4°C compared to 12°C (Xie et al., 2001). Although a slight delay in the expression of MRFs has been observed in some fish species, the intensive inhibition of myogenesis shown in the present study has not been observed at low temperature in fish. This is probably because temperature has a greater effect on homothermal animals than on poikilothermal ones. Our data on mouse skeletal muscle cells revealed several interesting new phenomena that have not been observed in fish. We speculate that the inhibition of myogenesis at low temperature might explain why homothermal animals keep their body temperature around 38°C in spite of the high energy consumption. This is of interest in comparing the temperature sensitivity of myogenesis between homothermal and poikilothermal animals.

Here we showed that the differentiation of mouse skeletal muscle cells may be regulated by temperature. We speculate that the regulation of cell differentiation by body temperature is a key to understanding the evolution of homothermal animals. In addition, manipulating temperature in living organisms is less invasive than administering chemical factors. Therefore, the regulation of myogenesis by temperature might be widely applicable in various fields, such as athletic training, stock raising, and the treatment of intractable muscular diseases such as muscular dystrophy.

ACKNOWLEDGMENTS

This work was supported by grants 18A-1 for Nervous and Mental Disorders and H19-kokoro-020 for Research in Brain Science from the Ministry of Health, Labour and Welfare, Japan and 17570058 and 14654174 from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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(Received June 23, 2008 / Accepted August 4, 2008)

リードスルー療法の最前線

Recent development of readthrough therapy for muscular dystrophy

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◎点変異により遺伝子エクソン内で未熟終止コドン(premature termination codon：PTC)が生じると，機能的な蛋白質が合成されず遺伝子欠損症状を呈するようになる．このナンセンス変異は Duchenne 型筋ジストロフィー(DMD)の場合，症例の 10～15%を占めるといわれている．たった 1 カ所に PTC が生じただけで，あたかも遺伝子全体が欠損したような致死的症状を呈するようになる．PTC 以外には遺伝子の塩基配列に問題がないならばその遺伝子を有効利用することで患者を救う方法はなかろうか．答えは“yes！”．それを実現すべく今，薬物を用いて PTC を翻訳過程で読み飛ばすこと(リードスルー)により正常機能蛋白質の発現を回復させようというリードスルー療法が注目されている．

Key word：筋ジストロフィー，ナンセンス突然変異，リードスルー療法，ゲンタマイシン，ネガマイシン

遺伝子エクソン内で点変異により未熟終止コドン〔たとえばグルタミンをコードする CAA の C が T に換わると終止コドンの TAA となる．このような変異をナンセンス変異とよび，形成された終止コドンを premature termination codon(PTC)とよぶ〕が生じると，機能的な蛋白質が合成されず遺伝子欠損症状を呈するようになる．このナンセンス変異は Duchenne 型筋ジストロフィー(Duchenne muscular dystrophy：DMD)の場合，症例の 10～15%を占め，嚢胞性線維症では一部のユダヤ系患者集団の 4 割，Hurler 症候群(ムコ多糖症)では 7 割を占めるといわれている．たった 1 カ所に PTC が生じただけで，あたかも遺伝子全体が欠損したような致死的症状を呈するようになる．

PTC 以外には遺伝子の塩基配列に問題がないならばその遺伝子を維持したまま有効利用することで患者を救うことはできないか？．答えは“yes！”．それを実現すべく今，薬物を用いて PTC を翻訳過程で読み飛ばすことにより正常機能蛋白質の発現を回復させようというリードスルー療法が注目されている．

ゲンタマイシンによるリードスルー

PTC を読み飛ばす？　そんな便利な薬物があるのかと疑う人も多いであろうが，実はあるのである．有名なストレプトマイシンやカナマイシンなどのアミノグリコシド系抗生物質がそれだ．この抗生物質は細菌の翻訳系を標的とする．これが働くと細菌の蛋白質合成が阻害され，抗菌活性を示す．しかし，この抗生物質は，真核生物においても mRNA の翻訳忠実度を低下させ，ときとしてコドンに指定されていないアミノ酸を組み込んだり(ミスコーディング)，あるいは翻訳終止コドンを読み越え(リードスルー)させることがある．

なかでもゲンタマイシン(図 1-A)はリードスルー活性が比較的高い．アメリカ・ペンシルバニア大学医学部の Lee Sweeney らは，DMD の疾患モデル動物である mdx マウス(DMD の原因遺伝子ジストロフィン上にナンセンス変異があり，機能的なジストロフィンを合成できずに筋変性を起こしているマウス)にゲンタマイシンを投与し，ジストロフィン mRNA 上の PTC をリードスルーさせることにより，機能をもったジストロフィンの蓄積および筋力の上昇させることに成功した[1]．

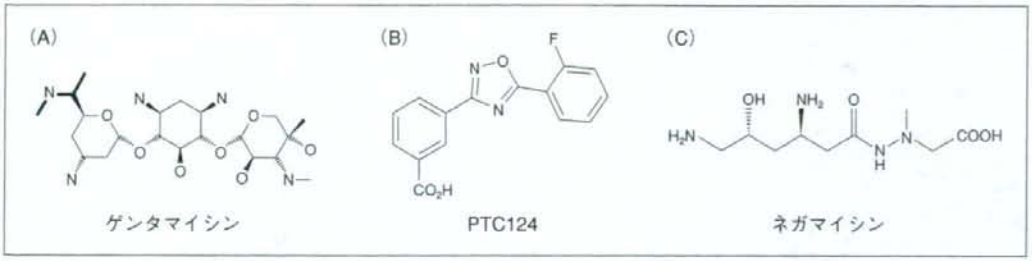

図 1　ゲンタマイシン(A)，PTC124(B)，ネガマイシン(C)の構造

リードスルー療法は，ナンセンス変異型筋ジストロフィーにおける筋細胞膜でのジストロフィンの役割を回復するための有効な選択肢のひとつである．たとえ少量のジストロフィンでも筋ジストロフィーの進行を遅延させ，患者の QOL の向上と延命をはかることが期待できるからだ．筋ジストロフィー以外にも適用可能な 2,400 種を超えるナンセンス変異型遺伝性疾患の存在が明らかになっている．しかし，ゲンタマイシンの血中濃度の安全域は狭く，強い腎毒性と聴覚毒性のため，投与が長期間に及ぶと予想される遺伝性疾患の治療薬としてのゲンタマイシンの使用は難しい．

PTC124によるリードスルー

ここではリードスルー薬物研究の 2 つの動向について述べる．

ひとつは，毒性が高いゲンタマイシンの代替物として Lee Sweeney らが推進している PTC124(図 1-B)という低分子を用いたリードスルー療法である．この PTC124 は PTC Therapeutics[2]が培養細胞系と無細胞系を用いたリードスルー活性のハイスループット検出系を使い，80 万種類の低分子化合物群から同定されたものである．PTC124 は経口投与で効果を発揮し，mdx マウスにおいては 4 週間の投与で正常対照マウスの 20〜25%量のジストロフィンの蓄積を観察している．また，使用する PTC124 の量は同程度の効果をもたらすゲンタマイシン量の 1/10〜1/100 ですむから安全性も高い[3]．健常人を被験者とする第Ⅰ相試験では毒性は認められず[4]，現在 DMD と Becker 型筋ジストロフィー患者を対象とする後期第Ⅱ相の臨床試験にかけられている．中間評価ではすでに薬理効果を見出しているが，確実な治療効果を得るために，より高用量での治験が行われている．また，PTC124 は脳血液関門を通過できるため，ナンセンス変異型の遺伝性脳疾患にもその有効性が期待される．

これらの概要は開発者である Lee Sweeney により，さる 2008 年 4 月にニューオリンズで開催されたシンポジウム“The Third New Directions in Biology and Disease of Skeletal Muscle”で発表された．それによると，12 例のナンセンス変異型 DMD 患者に 4 週間投与したところ，約半数の患者で投与 2 週間後には血中クレアチンキナーゼ(creatine kinase：CK．筋中に多量に存在する酵素．筋細胞の崩壊や筋細胞膜の透過性が亢進すると血中に漏出する．筋ジストロフィーの臨床的診断マーカーになっている)活性が低下，筋疲労も軽減し，症状の改善がみられたそうである．投与中止 4 週間後に CK 活性は上昇に転じたことからも，PTC124 投与は明らかな治療効果があったと判断される．この PTC124 は全米が注目しており，アメリカ筋ジストロフィー協会(MDA)や筋ジストロフィー親の会(PPMD)，食品医薬品局(FDA)，国立衛生研究所(NIH)などから多額の研究資金が投入されている．

ネガマイシンによるリードスルー

もうひとつは，著者らが行っているネガマイシン(図 1-C)，およびその関連物質を用いたリードスルー研究である．ネガマイシンは 30 年前にわが国で発見されたグラム陰性菌に対するジペプチド系抗生物質で，発見当初からミスコーディングとリードスルー惹起することが示されていた．そこで著者らは，このネガマイシンを mdx マウスへ連日皮下投与したところ，投与後 4 週間までに骨格

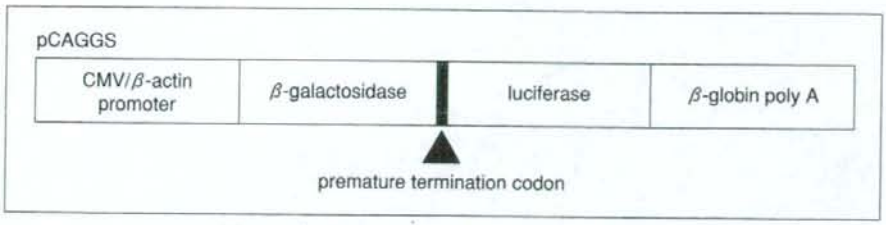

図 2　デュアルレポーター遺伝子コンストラクト

筋と心筋にジストロフィン蛋白質の蓄積(正常組織にみられるジストロフィン量の 10%)を認めた[5]．また，先天性筋ジストロフィーのモデル系においても高いリードスルー活性をもつことを示した[6]．ネガマイシンの毒性はゲンタマイシンよりはるかに低いため，長期間投与が予想されるリードスルー療法薬として有望である．しかし，ネガマイシンは未承認抗生物質であり，ゲンタマイシンに比べ実用化へのハードルは高い．

さらに問題なのは，現在ではネガマイシンの入手がほぼ不可能なことである．そこで著者らは，ネガマイシンをリード物質としたあらたな薬物候補の探索に着手した．mdx マウスを薬効評価系に使うと含量が少なく，分子量が大きいジストロフィンの検出定量性が問題となる．そこでリードスルー活性検出の評価に特化した解析系の開発からはじめた．著者らは，ルシフェラーゼ遺伝子の前方に PTC を挟んでβ-ガラクトシダーゼ遺伝子を結合し，β-アクチンプロモーターをつけたデュアルレポーター遺伝子をつくり(図 2)，これを導入したトランスジェニックマウスの作製に成功した．このマウスは骨格筋・心筋組織において普段はβ-ガラクトシダーゼのみを発現しているが，リードスルーが起こるとルシフェラーゼも発現する．そのため，このマウスを使えばルシフェラーゼ活性とβ-ガラクトシダーゼ活性の比を求めることで，リードスルー活性を定量化することができる．また，投与経路・量と標的組織別感受性(リードスルー活性と薬物動態)を同時に評価することが可能なため，単に薬効においてのみ注目したスクリーニングではなく，重篤な副作用をもたない化合物を特定することもできる．

著者らはアメリカ・メリーランド大学薬学部の MacKerell 教授とともに *in silico* 検索を行い，105 万種類の低分子化合物データベースから，ネガマイシンに類似した三次元構造をもつ 29 種の分子を見出した．それらのうち 17 種を入手し，トランスジェニックマウスに皮下投与したところ，5 種類のリードスルー活性を有する物質を特定した(国際特許出願中)．そのなかの化合物#2 は内服によってもリードスルー惹起効果を示し，mdx マウスや DMD 患者由来培養細胞を用いた生化学的・免疫組織化学的解析結果も良好で，安全性も高いことから，PTC124 と同様にリードスルー療法薬として有望であると考えている(図 3)．また，ネガマイシン誘導体[7]や未承認アミノグリコシド系抗生物質からもリードスルー惹起活性をもつ分子を数種特定している．

リードスルー薬物の経皮投与

いまのところ，ゲンタマイシンや PTC124，ネガマイシンや化合物#2 においては自然終止コドンのリードスルーは認められていないが，リードスルーを惹起することにより生存に必須な蛋白質をコードした mRNA のミスコーディングや自然終止コドンのリードスルーも起こす可能性がある．また，それ以外の副作用についても未知なところが多い．薬物を患者の QOL の改善につながる筋肉に特異的に薬物を分配できれば，全身投与に比べて治療効率の向上と副作用の軽減につながると考えられる．さらに，注射による投与を回避できれば，注射による苦痛もなくなる．

そこで著者らは，通常は皮膚を透過しない薬物に対する経皮的投与法を開発した．用いたのは，そのままでは経皮的投与によるリードスルーは起こらないゲンタマイシンと，先述したトランスジェニックマウスと体毛がないヘアレスマウスである．まずトランスジェニックマウスを剃毛し，

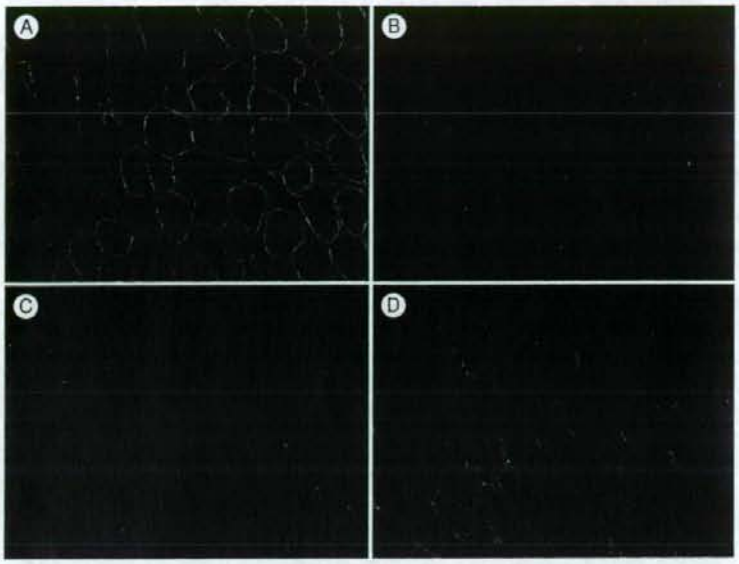

図 3　マウス骨格筋のジストロフィン免疫染色像
A：正常 B10 マウス，B：未投与 mdx マウス，C：ゲンタマイシンを皮下投与した mdx マウス，D：化合物#2 を経口投与した mdx マウス．

皮膚の透過性を増すために簡単な前処理を施した．その後，市販のゲンタマイシンクリームを連日塗付し，7 日後に塗布した皮膚直下にある筋肉におけるリードスルー活性を測定した．薬物処理していない皮膚にゲンタマイシンを塗布したマウスを対照として測定したところ，皮膚に前処理を受けたマウスのみ高いリードスルー活性を示した．剃毛による皮膚の微小な擦過傷を回避するためヘアレスマウスを用い，前処理あるいは対照として，処理していないマウスの皮膚にゲンタマイシンクリームを塗布し，血中と骨格筋内におけるゲンタマイシン分子の定量を HPLC と質量分析により行った．その結果，皮膚を前処理した後ゲンタマイシン塗擦したマウスのみ，その血中と筋組織中に高濃度のゲンタマイシンを認めた．

経皮吸収型薬物送達法は，初回通過効果(経口投与した薬物は消化管で吸収され，門脈を経由して肝で代謝されてから代循環血液中に到達するため，到達する割合と速度が低下する現象)や消化管障害を回避でき，血中濃度の持続化や苦痛の解消，患者コンプライアンスの向上を提供できる点で魅力的である．この経皮投与法はリードスルー療法のみならず，他の薬物療法にも適用可能なため大きな社会的成果が期待される．

リードスルー療法以外の薬物療法

そのほか，ニューオリンズでの会議で発表されたなかで注目されるものがいくつかあったので，ここに簡単に紹介したい．

DMD の場合，欠損しているジストロフィンをリードスルーにより補充する以外に，ユートロフィン(ジストロフィンの胎児型アイソフォーム．ヒト第 6 染色体にコードされているためジストロフィン遺伝子に異常がある場合でもユートロフィン遺伝子は正常である．胎児ではジストロフィンの代りに筋細胞膜直下の裏打ち蛋白質として存在するが，成体では神経筋接合部にのみ局在する)の発現を上昇させて，症状悪化を回避する戦略も考えられる．イギリスの Davies らは，ユートロフィン・プロモーターにルシフェラーゼ遺伝子を結合したレポーター遺伝子をつくり，その遺伝子を導入した細胞培養系を用いてユートロフィン発現惹起活性を検出する系を確立した．この細胞に多数の低分子化合物をロボットを用いてハイスループット実験を行い，ユートロフィン発現を惹起する薬物候補 SMT C1100 を見出した．これを mdx マウスに投与するとユートロフィン mRNA 量が 2 倍に増加し，筋変性の指標である再生筋線維数と血中 CK 値がともに低下し，筋組織における線

維化，脂肪変性と炎症の減少がみられた．さらに，プレドニゾロンとの併用では筋疲労の軽減もみられた．この薬物の長期的副作用は不明であるが，注目すべき結果である．彼らは Summit PLC[8]というベンチャー会社を起こし，これを薬物として実用化すべく，2008 年中期には第 I 相臨床試験を行う予定である．

また，Biglycan の補充療法も提案された．この Biglycan は筋細胞膜に局在するαサルコグリカンとγサルコグリカンを結合する役割を担う細胞外基質で，筋細胞膜を構成する蛋白質やシグナル伝達の調整を行っている．Biglycan 欠損マウスでは軽度な筋変性を生じる．mdx マウスではジストロフィン関連糖蛋白質複合体の形成が阻害され，筋基底膜との結合に障害が生じているため，Biglycan も不足している．アメリカ・ブラウン大学医学部の Fallon らは，Biglycan 欠損マウスに Biglycan を腹腔注射により補充すると，ジストロフィン糖蛋白質複合体が正常に形成され，筋変性が軽減することを示した[9]．さらに，ヒトリコンビナント Biglycan を mdx マウスに腹腔内投与すると 5 週間後に対照 mdx マウスに比べ筋力低下が抑制され，ユートロフィン蛋白質の含量が 2.5 倍増加し，横隔膜において筋変性が軽減し，浸潤細胞も減少するなど治療効果を示した．また，ユートロフィンノックアウトマウスと mdx マウスとの交配で作製したダブルノックアウトマウスにおいても，Biglycan 投与の効果は認められた．今後，この Biglycan 補充療法はおおいに期待できそうである．

おわりに

本稿ではリードスルー療法を中心に薬物による遺伝性疾患，とりわけ DMD の治療開発についての最近の知見を概観した．リードスルー薬物はいまのところ PTC124 の独壇場になりつつあるが，PTC124 のみでは多様なナンセンス変異型遺伝性疾患への臨床応用は個々の患者の PTC に対する有効性，PTC 周辺の塩基配列の違いによる特異性および副作用の蓄積など，不安要素がある．そこで著者らの見出した化合物#2 のように，できるだけ多くの薬物候補分子の探索を今後とも続けていく必要がある．また，Biglycan の補充療法にみられるように，DMD の治療には筋変性メカニズムの基本的理解により筋変性を抑制するあらたな分子標的が浮かび上がってくる場合もある．原因遺伝子の欠損が，いかにして病状を呈するに至るかの解明は今後も続けていかなければならない．

DMD の根本治療にはまず正常ジストロフィン遺伝子あるいは短縮型ジストロフィンの遺伝子導入が推進されるべきものである．また，最近注目されている幹細胞による移植治療も有望である．点変異型の DMD にはリードスルーやエクソンスキップによる治療も適応できる．さらに，抗炎症薬/ステロイドや蛋白質分解酵素阻害剤，抗酸化物質などによる筋ジストロフィー症状の軽減も期待できる．DMD では患者ごとに遺伝子異常のパターンが異なる．患者ごとに適した戦略を組み合わせることで DMD の効果的治療につながると思われる．

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抗生物質とエキソン・スキップによる筋ジストロフィーの治療

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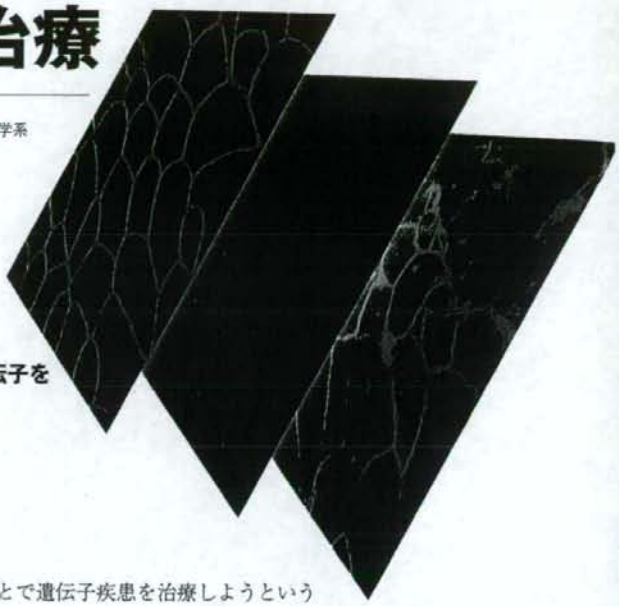

**新生男児3500人に一人の割合で出現する
デュシェンヌ型筋ジストロフィーは
10代の少年を襲う致死的遺伝子疾患である。
では，ジストロフィンの遺伝子導入以外に
デュシェンヌ型筋ジストロフィーを治す方法はないのか？
せっかく存在する患者自身の欠陥があるジストロフィン遺伝子を
有効活用しながら治療に結びつけることはできないのか？
ここでは近年，注目されているリードスルー療法と
エキソン・スキップ療法の進展状況について紹介しよう。**

デュシェンヌ型筋ジストロフィー（Duchenne muscular dystrophy：DMD）の原因タンパク質，ジストロフィンが発見されて20年が経った。発見当時は20年後にはDMDの遺伝子治療も実現しているであろうと予想されていたが，いまだ実現には至っていない。

残念ながらDMDに限らず，原因遺伝子が解明されても，遺伝子疾患を治療できた例は多くはなく，むしろ事故が目立つのが現状である。1999年，アメリカ・ペンシルベニア大学でアデノウイルスをベクターにして遺伝子治療を受けた18歳の男性患者が4日後に死亡した。遺伝子導入に使用した大量のウイルスに対する免疫ショックがおきたためであった。また，2002年にはフランスでレトロウイルスをベクターにした遺伝子治療を受けた免疫不全症の患者が白血病を発症した。これは患者のゲノムへのウイルス由来遺伝子の挿入部位に大きな問題があったと考えられている。

一方，遺伝子疾患をもつ患者から分離した幹細胞ないしはiPS細胞（人工多能性幹細胞）に対して正常遺伝子を導入し，それを同じ患者に再移植することで遺伝子疾患を治療しようという戦略もある。患者自身の幹細胞を使えば免疫学的，倫理的問題を回避できる。しかし，幹細胞の分化運命の制御や体内に移植された後の組織構築の促進，さらに腫瘍化の回避などにはいまだ多くの課題が残されている。

これらの遺伝子導入療法を補完する遺伝子疾患の治療戦略として注目されているのが，薬物によるリードスルー療法とアンチセンスオリゴヌクレオチドによるエキソン・スキップ療法である。

リードスルー療法とは？

mRNAの翻訳中に原因遺伝子上に点突然変異によって生じた未熟終止コドン（premature termination codon：PTC）がくると，リボソームは正常の翻訳終止点と同様に認識し，遊離因子の作用によりmRNAを遊離し，タンパク質合成を終了してしまう。したがって，機能をもった全長タンパク質が合成されず，種々の遺伝子疾患が生じる。ほとんどの遺伝子疾患症例の5～15%はPTCによる翻訳の中断（ナンセンス突然変異）が原因といわれている。さらに嚢胞

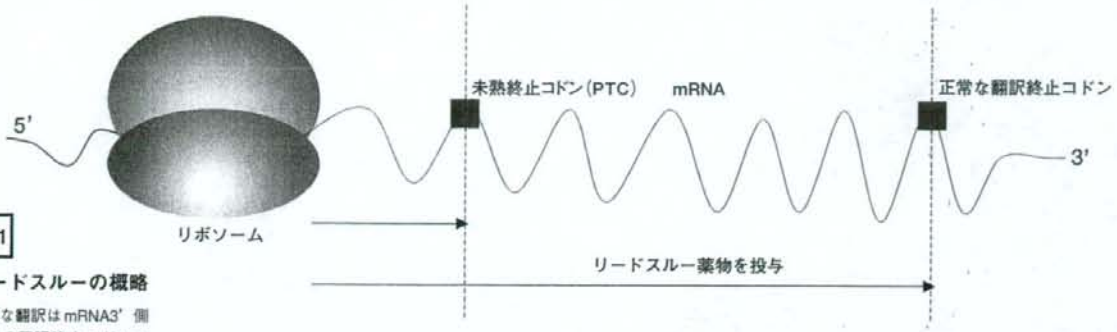

図1

リードスルーの概略

正常な翻訳はmRNA3'側にある翻訳終止コドンで終結する。もし，ナンセンス突然変異により未熟終止コドン（PTC）が生じると翻訳はそこで中断し，鎖長が短いタンパク質を生じる。多くの場合，このタンパク質は機能をもたず，細胞内で分解される。ゲンタマイシンやネガマイシンなどのリードスルー薬物はrRNAのAサイトに結合し，翻訳の効率や忠実度を低下させる。ときとして，PTC部位に他のtRNAが付き，翻訳を続行させることがある。これがリードスルーである。

性線維症（Cystic Fibrosis：CF）では，ナンセンス突然変異は症例の40%に上るといわれている。もし，薬物によりPTCを読み越えさせること（リードスルー）ができれば，機能的なタンパク質の合成が回復し症状の改善が期待される。これがリードスルー療法である（図1）。

リードスルーを惹起する薬物はどんなものがあるのだろうか。まず，その候補として挙げられているのは，ゲンタマイシンなどのアミノグリコシド系抗生物質である。この抗生物質は，細菌のタンパク質翻訳系を標的とし，翻訳忠実度を下げて異なったアミノ酸の挿入（ミスリーディング）[1-3]や終止コドンの読み越え（リードスルー）[4,5]を誘導することで3'非翻訳領域まで翻訳が進み，正常より長いタンパク質分子を合成させることが大腸菌，酵母や無細胞翻訳系を用いて示されている。このリードスルー現象が，ナンセンス突然変異による遺伝子疾患をもつ患者の細胞内でもおきれば，PTCを翻訳過程で読み越えて機能的タンパク質を合成させることにより，治療法に応用できると予想できる。

候補薬物としてのゲンタマイシン

1997年，アメリカ・アラバマ大学のBedwellらは，CF患者由来細胞においてCF原因遺伝子であるCF膜貫通制御因子（CFTR）遺伝子上のPTCを，ゲンタマイシンを用いてリードスルーさせることに成功した[6]。さらにアデノウイルスベクターによる感染事故がおきた同じアメリカ・ペンシルベニア大学で，Barton-DaviesとLee H. Sweeneyらは，ゲンタマイシンをDMDのモデル動物であるmdxマウス（ジストロフィン遺伝子のエキソン23にナンセンス突然変異がある）に投与すると欠損していたジストロフィンの発現が回復し，筋力が上昇したことを報告した[7]。しかし，アミノグリコシド系抗生物質は強い腎毒性と聴覚毒性をもち，短期間の抗菌剤としては使用できるものの長期間にわたる遺伝子治療薬として用いるには強い副作用が問題となることが予想される。

Made in Japanのネガマイシン

このゲンタマイシンと同様にリードスルー活性をもち，より副作用が少ない抗生物質はないだろうか。幸いなことに，1970年，わが国の微生物化学研究所で発見されたジベプチド系抗生物質ネガマイシンが，大腸菌においてミスコーディングやリードスルーをおこすことが証明されていた[8]。われわれは早速，ネガマイシンを入手し，mdxマウスへの投与実験をしたところ，ゲンタマイシン同様，ネガマイシン投与群の骨格筋と心筋組織においてジストロフィンの全長タンパク質の合成と蓄積を認めた（図2）。

ゲンタマイシンの場合，問題になる強い聴覚毒性がネガマイシンにもみられるかを知るため，ネガマイシンあるいはゲンタマイシンの投与を受けたマウスの聴性脳幹反応テストを実施した。その結果，ゲンタマイシン投与群において聴力のいちじるしい低下を認めたが，ネガマイシン投与群では正常レベルの聴力を維持しており，聴覚毒性は認められなかった。さらにネガマイシンのLD_{50}はマウスではゲンタマイシ

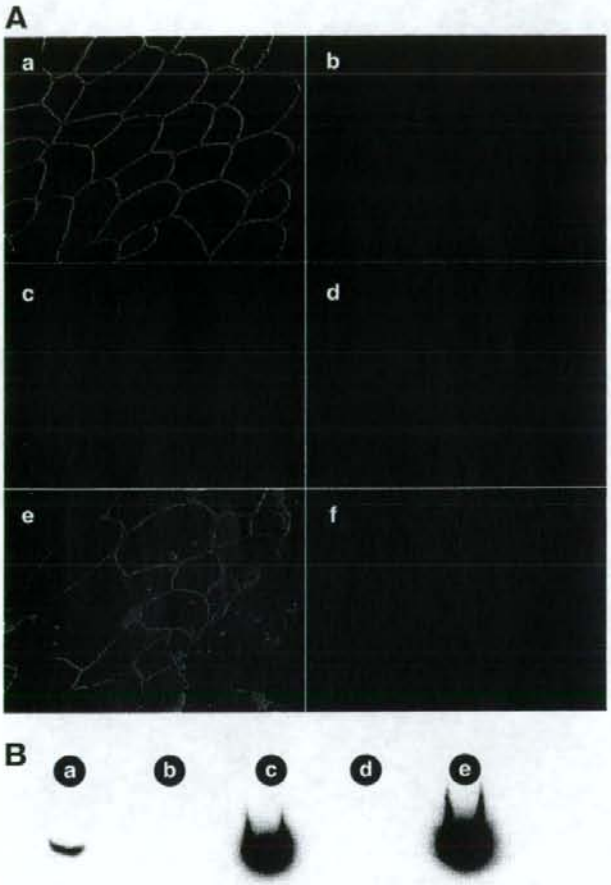

図3 ネガマイシンはmdxマウスの骨格筋にジストロフィン合成を促す

A. ジストロフィンの蛍光抗体染色像（a, c, e）とエバンスブルーによる生体染色像（b, d, f）

（a）1%エバンスブルー溶液0.2mLをB10対照マウスに静脈内注射により生体染色し，24時間後に採取した前頸骨筋から凍結横断切片（10μm厚）を作製し，無固定のまま抗ジストロフィンポリクロン抗体とFITC標識された抗ウサギIg抗体を用いて染色した。ジストロフィンは筋細胞膜直下にある裏打ちタンパク質であり，筋ファイバーの全周を染める。

（b）aと同じ切片を560nmで励起し，580nmにて観察したエバンスブルーの蛍光像。正常な筋ファイバーの細胞膜にはエバンスブルーは透過しない。小静脈と一部の細胞間質だけにエバンスブルーの赤色蛍光が認められる。

（c）エバンスブルーをaと同様に静脈内注射されたmdxマウスの前頸骨筋の凍結横断切片を抗ジストロフィン抗体で染色した。mdxマウスはジストロフィン遺伝子にナンセンス突然変異があり，機能的ジストロフィンの発現と細胞膜直下への蓄積は見られない。

（d）cと同じ切片のエバンスブルー蛍光像。筋ジストロフィー変化により膜変性をおこした筋ファイバーの細胞膜はエバンスブルーを透過し，ファイバー細胞質が染色されている。

（e）2週間，連日ネガマイシンを皮下投与されたmdxマウスの前頸骨筋におけるジストロフィンの蛍光抗体染色像。f, eと同じ切片におけるエバンスブルー蛍光像。ネガマイシンによりジストロフィンが回復した筋ファイバーにはエバンスブルーによる染色は認められない。白線は100μmを示す。aからeまで同倍率で観察した。

B. B10対照マウス，無処理mdxマウスおよびネガマイシンを投与されたmdxマウス前頸骨筋抽出液の抗ジストロフィン抗体によるイムノブロット像

（a）ネガマイシンを投与されたmdxマウス下肢骨格筋抽出液（タンパク質量20μg）。

（b）サンプルバッファーのみ。

（c）aと同じサンプルを10倍量泳動した。

（d）サンプルバッファーのみ。

（e）対照B10マウスの下肢骨格筋抽出液（タンパク質量20μg）。バタンは発光基質を用いて可視化した。文献[9]より引用。

ンの100分の1程度であった。質量分析法によりネガマイシンはゲンタマイシンと同様，リボソームRNAのAサイトに結合することがわかり，これによりmRNAの翻訳忠実度の低下をもたらすことが予想された[9]。その後，ネガマイシンは先天性筋ジストロフィー患者の筋細胞に対し，ゲンタマイシンの場合より高いリードスルー活性をもつなど，ネガマイシンはゲンタマイシンを代替するリードスルー薬としての有効であることが示された[10]。

しかし，残念ながらネガマイシンは未承認抗生物質であり，しかも収量も低く精製も困難な抗生物質である。ネガマイシンの生産は，抗生物質開発の熱が冷めた現在の日本ではむずかしい状況であった。しかし最近，東京薬科大学の林らのグループにより効率が高い全合成系が確立されたことは特記に値する[11]。われわれは，*in silico*検索で創薬開発を進めているアメリカ・メリーランド大学のA. マッケレル教授と共同で，ネガマイシンと立体構造が類似した化合物を105万種類の低分子量化合物の分子構造データライブラリからコンピュータ上でサーチし，28種類の化合物にたどり着いた[12]。

リードスルー活性測定用マウスの開発

一方，ジストロフィンを指標にする場合，ジストロフィンが42万ダルトン以上の巨大分子であるためイムノブロットでの転写効率は低く，その存在量は他の筋タンパク質に比べて少な