

Fig. 1 Diagnostic algorithm of pneumonia during tocilizumab therapy

Elevation of serum lipid concentrations

In clinical trials, serum lipid levels are often elevated during TCZ therapy [4–6, 19–22]. Serum total cholesterol increased in 38% of patients on TCZ monotherapy in one Japanese study [5] and in 23% of the patients given a combination of TCZ and nonbiologic DMARDs in a European study [22]. However, total cholesterol levels stabilized in the upper normal range in most cases. Furthermore, the atherogenic index was not altered in these cases due to accompanying increases in high-density lipoprotein [5, 22].

Cardiovascular complications were rarely reported during TCZ clinical trials, although the observation periods were relatively short [5, 22]. Statin therapy is recommended when lipid levels increase above normal range. These phenomena are probably due to suppression of inflammation, which can also be observed under anti-TNF treatment. Nevertheless, elevated serum lipids during TCZ treatment should be monitored during postmarketing surveillance since IL-6 signaling might also directly affect lipid metabolism.

Transient neutropenia

Neutropenia has been reported with TCZ treatment [6, 19–22]. Neutrophil counts below $1,000/\text{mm}^3$ were recorded,

although not associated with infection. The transient nature of these changes indicates that margination of neutrophils from the circulation in the bone marrow might be responsible [19].

Combination with other DMARDs

Since the clinical trials of TCZ in Japan were designed as monotherapy treatment [5], this drug has been approved only for use without concomitant DMARDs. Clinical trials in Western countries have shown the efficacy of TCZ treatment when combined with conventional DMARDs, particularly MTX. Elevated levels of hepatic transaminases were detected more frequently in the TCZ groups in these trials [19, 21], although their levels were less than threefold the upper limits of normal in the majority of patients and returned to normal with repeated treatment. No such findings were reported in Japanese trials [4, 19, 22]. Monitoring of serum liver enzymes is recommended during TCZ treatment, especially when the latter is combined with MTX.

Infusion reactions

Similar to other biologic agents, TCZ can induce infusion reactions during or after the treatment day, although most reactions are mild and transient [4–6, 19–22]. Infusion

reactions were observed in 11 out of 157 patients in the SAMURAI trial [5]. Sleepiness, headache, and increased blood pressure were observed but they were transient in nature. However, preparation for severe infusion reactions is recommended in case of emergencies; airway maintenance, oxygen inhalation, subcutaneous epinephrine, and intravenous corticosteroids should be available at bedside.

Surgery

The effect of IL-6 on wound healing remains to be clarified [34], although it is possible that wound healing might be delayed under TCZ therapy. Moreover, the signs and symptoms of surgical-site infection might be masked in patients treated with TCZ, as discussed above. CRP levels were completely normalized in patients with stable serum TCZ levels [35]. At present, it is advisable that surgery is postponed in patients on TCZ treatment until reduction of drug level in peripheral blood, i.e., deferment of surgery to at least 14 days after the last infusion of the drug.

Pregnancy and lactation

There are no data available on the safety of TCZ in pregnancy and lactation. IL-6 was reported to reduce recurrent abortion in an animal model [36], however the effect of TCZ on embryonic and fetal development is unknown. Therefore, it is currently not recommended to administer TCZ to pregnant women.

Malignancy

It remains unclear whether blocking of IL-6 signaling affects the progression of malignancy [37]. IL-6 induces cachexia in cancer patients, but might also inhibit proliferation of certain types of cancer cells [38]. Administration of TCZ to patients with present or recent history of malignancy or precancerous lesions should be avoided at present, in line with recommendations for anti-TNF agents.

Summary

TCZ is a novel therapeutic option for RA patients who exhibit inadequate response to nonbiologic or biologic DMARDs. Treatment with this drug prevented structural joint damage in Japanese clinical trials. However, the safety profile of TCZ is not yet defined since the numbers of patients involved in the clinical trials are insufficient to detect infrequent but potentially serious adverse effects. Therefore, the JCR has developed tentative guidelines for

the proper use of TCZ, and postmarketing surveillance is underway according to these preliminary guidelines.

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Extended report

Long-term safety and efficacy of tocilizumab, an anti-IL-6 receptor monoclonal antibody, in monotherapy, in patients with rheumatoid arthritis (the STREAM study): evidence of safety and efficacy in a 5-year extension study

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ABSTRACT

Objectives: To evaluate the safety and efficacy of 5-year, long-term tocilizumab monotherapy for patients with rheumatoid arthritis.

Methods: In an open-label, long-term extension trial following an initial 3-month randomised phase II trial, 143 of the 163 patients who participated in the initial blinded study received tocilizumab monotherapy (8 mg/kg) every 4 weeks. Concomitant therapy with non-steroidal anti-inflammatory drugs and/or oral prednisolone (10 mg daily maximum) was permitted. All patients were evaluated with American College of Rheumatology (ACR) improvement criteria, disease activity score (DAS) in 28 joints, and the European League Against Rheumatism response, as well as for safety issues.

Results: 143 patients were enrolled in the open-label, long-term extension trial and 94 (66%) patients had completed 5 years as of March 2007. 32 patients (22%) withdrew from the study due to adverse events and one patient (0.7%) due to unsatisfactory response. 14 patients withdrew because of the patient's request or other reasons. The serious adverse event rate was 27.5 events per 100 patient-years, with 5.7 serious infections per 100 patient-years, based on a total tocilizumab exposure of 612 patient-years. Of the 88 patients receiving corticosteroids at baseline, 78 (88.6%) were able to decrease their corticosteroid dose and 28 (31.8%) discontinued corticosteroids. At 5 years, 79/94 (84.0%), 65/94 (69.1%) and 41/94 (43.6%) of the patients achieved ACR20, ACR50, and ACR70 improvement criteria, respectively. Remission defined as DAS28 less than 2.6 was achieved in 52/94 (55.3%) of the patients.

Conclusion: In this 5-year extension study, tocilizumab demonstrated sustained long-term efficacy and a generally good safety profile.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by persistent synovitis and progressive joint damage.¹ Although the causes of RA are not fully understood, constitutive overproduction of IL-6, a multifunctional cytokine that regulates the immune response, inflammatory reaction and bone metabolism, is thought to play a major pathological role in RA.²

Tocilizumab is a humanised anti-human IL-6 receptor monoclonal antibody,² which has been demonstrated to improve the signs and symptoms of RA³⁻⁹ and prevent radiographic progression¹⁰ in previous clinical trials. Those controlled trials

provided evidence for a rapid reduction in disease activity in response to tocilizumab in patients with active RA as measured by American College of Rheumatology (ACR) responses, disease activity scores (DAS) and a modified health assessment questionnaire (MHAQ).⁵⁻⁹ The efficacy was dose related and 8 mg/kg tocilizumab provided a marked clinical benefit. The success in the treatment of patients with RA using tocilizumab confirmed that IL-6 plays an important pathological role in RA, and further studies were therefore required to determine the long-term safety and efficacy of tocilizumab treatment. We report here the safety and efficacy of tocilizumab in a 5-year long-term extension study.

METHODS

Patients

This study was registered with <http://www.clinicaltrials.gov> (NCT00144651). The study protocol was approved by the Ministry of Health, Labor and Welfare of Japan and by the ethical committee of each institute, and patients gave their written informed consent.

The eligibility criteria and the study design of the initial 12-week, randomised, double-blind, placebo controlled study have been reported previously.⁵ Briefly, eligible patients were 20 years of age or older and fulfilled the 1987 criteria for RA of the American Rheumatism Association¹¹ with a disease history of longer than 6 months. All subjects had been insufficient responders to treatment with at least one disease-modifying anti-rheumatic drug (DMARD) or immunosuppressant. Patients had active disease at the time of enrollment into the initial controlled trial, as defined by the presence of six or more swollen joints, six or more tender joints and one of the following two criteria: a Westergren erythrocyte sedimentation rate (ESR) of at least 30 mm/h or a C-reactive protein (CRP) level of more than 1.0 mg/dl. Patients receiving prednisolone (10 mg daily maximum) and/or non-steroidal anti-inflammatory drugs (NSAID) were eligible if the dose had not increased during the washout period of 1 month. Doses of both medications remained stable during the blinded study period of 12 weeks. Patients who had received tocilizumab or placebo twice or more were given the opportunity to receive tocilizumab in this open-label extension trial.



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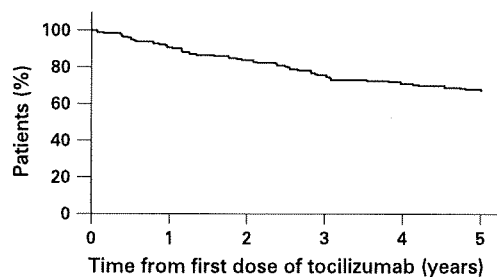


Figure 1 Kaplan-Meier estimate of the probability of the patients remaining on study. Treatment time was calculated beginning with the first infusion of tocilizumab at any dose, excluding the time receiving placebo.

In the extension study, the use of prednisolone (10 mg daily maximum) and one NSAID was permitted. Sexually active premenopausal women were required to have a negative urine pregnancy test at entry and to use effective contraception during the study period.

Treatment

Patients were randomly assigned to receive either placebo, or 4 or 8 mg/kg body weight of tocilizumab every 4 weeks in the initial blinded 12-week trial. In the first 12 weeks of the open-label extension study, patients received 8 mg/kg tocilizumab every 4 weeks and thereafter dose reduction and treatment interval changes (minimum 2 weeks) were allowed.

Efficacy assessments

Disease activity was assessed at baseline and at every visit during the initial blinded trial and the first 12 weeks of the extension study, and thereafter every 3 months. All patients were evaluated with ACR improvement criteria, DAS28 and the European League Against Rheumatism response. The DAS28 was calculated using the ESR. Clinical assessments included the following: complete counts of swollen and tender joints (49 joints evaluated; cervical spine and hips evaluated only for tenderness); physician's and patient's global assessment of disease status, on a visual-analogue scale from 0 (asymptomatic) to 100 (severe symptoms); patient's assessment of pain on a scale from 0 (no pain) to 100 (severe pain); functional disability measured with a MHAQ; ESR and CRP levels.¹² Treatment time was calculated beginning with the first infusion of tocilizumab, excluding the time receiving placebo.

Safety assessments

Safety was assessed for all patients who received at least one dose of tocilizumab in the extension study. Serious adverse events (SAE) were defined as events that were fatal or life-threatening, leading to permanent or significant disability or incapacity, a congenital anomaly or birth defect, or requiring prolonged inpatient hospitalisation. Adverse events were classified using the Medical Dictionary for Drug Regulatory Affairs (MedRA version 8.0).

Statistical analysis

Patients who had remained in the study and had completed visit reports were analysed. No imputation was used for missing data. A paired t test was employed to detect statistically significant differences in disease activity and functional outcomes from baseline. Statistical analyses were performed with

Table 1 Demographics and baseline clinical characteristics of patients with RA who received tocilizumab at any time during the blinded period or open-label extension of the tocilizumab study

	Tocilizumab (n = 143)
Demographics	
Age, years (SD)	54.3 (11.1)
No of men/no of women	34/109
Clinical characteristics	
RA duration, years (SD)	9.9 (8.4)
No of failed DMARD, mean (range)	4.5 (1–11)
Functional class,* I/II/III/IV	10/93/40/0
RA stage,* I/II/III/IV	3/34/56/50
Tender joint count, 0–49 scale (SD)	20.3 (10.3)
Swollen joint count, 0–46 scale (SD)	14.5 (8.7)
ESR, mm/h (SD)	68.7 (29.9)
CRP, mg/dl (SD)	4.7 (3.3)
DAS28 (SD)	6.7 (1.0)

Values are mean (SD) unless stated otherwise. The data were calculated from the baseline of the double-blind trial (4 mg/kg group, 8 mg/kg group) and from the extension trial (placebo group). *Rheumatoid arthritis (RA) functional status determined by American College of Rheumatology criteria. RA stage determined by Steinbrocker's criteria. CRP, C-reactive protein; DAS28, disease activity score in 28 joints; DMARD, disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate.

SAS version 8.2 TS2M0. The continuation rate, defined as the cumulative percentage of patients still receiving medication, was analysed using the Kaplan-Meier method. Analysis of adverse events was performed with the person-year method.

RESULTS

Characteristics of the patients

A total of 143 patients was enrolled in the open-label, long-term extension trial; 108 patients (76%) had completed 3 years and 94 patients (66%) had completed 5 years, as of March 2007 (fig 1). The median duration of treatment with tocilizumab was 66.7 months (range 0.95–73.2).

Thirty-two patients (22%) withdrew due to adverse events. Only one patient (0.7%) withdrew due to unsatisfactory response. Other reasons for withdrawals were as follows: eight for patient's personal requests; one for the emergence of anti-tocilizumab antibodies and five for other reasons.

The baseline demographic and clinical data are summarised in table 1. The patients' mean age was 54 years and the mean disease duration was 9.9 years. Patients had very active disease at baseline, in terms of the increased number of tender and swollen joint counts and elevated ESR of 68.7 mm/h and CRP levels of 4.7 mg/dl. Furthermore, the baseline DAS28 was 6.7.

Safety

A total of 148 SAE was reported in 77 patients (53.8%) for an overall rate of 27.5 events per 100 patient-years. Table 2 shows SAE (occurring in at least 1% of patients). Joint surgery related to RA was the most common SAE and occurred in 20 patients (14.0%). In addition, a variety of musculoskeletal disorders was reported as SAE, which were classified as not related to tocilizumab.

Serious infections were reported in 25 patients (17.5%) at a rate of 5.7 events per 100 patient-years. The most frequently reported infections were as follows: pneumonia (nine patients, 1.5 events per 100 patient-years); herpes zoster (seven patients, 1.1 events per 100 patient-years); acute bronchitis (five patients,

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Table 2 Serious adverse events observed in at least 1% of patients

SAE	No (%)
Any SAE	77 (53.8)
Joint surgery	20 (14.0)
Pneumonia	9 (6.3)
Herpes zoster	7 (4.9)
Tendon rupture	5 (3.5)
Humerus fracture	4 (2.8)
Spinal osteoarthritis	3 (2.1)
Femoral neck fracture	3 (2.1)
Joint dislocation	2 (1.4)
Back pain	2 (1.4)
Lumbar spinal stenosis	2 (1.4)
Bronchitis acute	2 (1.4)
Pyelonephritis	2 (1.4)
Brain stem infarction	2 (1.4)
Cataract	2 (1.4)
Pneumothorax	2 (1.4)
Liver function abnormality	2 (1.4)

SAE, serious adverse event.

0.8 events per 100 patient-years) and pyelonephritis (three patients, 0.5 events per 100 patient-years).

Four malignancies were reported in four patients (2.8%; 0.7 events per 100 patient-years). The types of malignancies were bladder cancer, breast cancer, large intestine carcinoma and intraductal papilloma.

Temporary prolongation of treatment intervals with tocilizumab was observed throughout the study. Although 163 events of prolonged intervals of 8 weeks or more occurred, the majority of the prolongation of intervals was due to transition from the randomised study to the extension study (median interval of the transition was 10.1 weeks). No particular adverse events were reported when tocilizumab was re-administered except for one patient with a severe infusion reaction. The patient had received 4 mg/kg tocilizumab in the initial 3-month trial, and IgE anti-tocilizumab antibodies appeared at the second infusion of the extension trial. Two more patients were positive for anti-tocilizumab antibodies, when tocilizumab was not detectable in their blood. No adverse event was reported related to the anti-tocilizumab antibodies.

Mean non-fasting total blood cholesterol increased after treatment initiation and stabilised (mean values 185 mg/dl at baseline; 220 mg/dl at 12 months; 214 mg/dl at 60 months; fig 2A). A total of 112 patients experienced total cholesterol abnormalities at at least one point and 15 patients had abnormal values at baseline. Thirty-nine patients (34.8%) were treated with statins, including two patients who had started statin treatment before the trial. There were no cardiovascular SAE related to tocilizumab except for ischaemic heart disease reported in one patient whose total blood cholesterol increased from 168 mg/dl at baseline to 227 mg/dl without statin treatment. The patient also had the risk factor of diabetes mellitus.

Mean neutrophil counts decreased but remained within the normal range (fig 2B). Grade 2 neutropenia was observed in 17 patients and grade 3 in nine patients. All the events were transient, and no patients experienced febrile neutropenia or withdrew as a result of neutropenia.

Mean aspartate aminotransferase (AST) and alanine aminotransferase (ALT) increased slightly, but remained roughly within the normal ranges (fig 2C). Grade 2 or higher increases in AST and ALT occurred in nine (6.3%) and 14 (9.8%) of 143 patients,

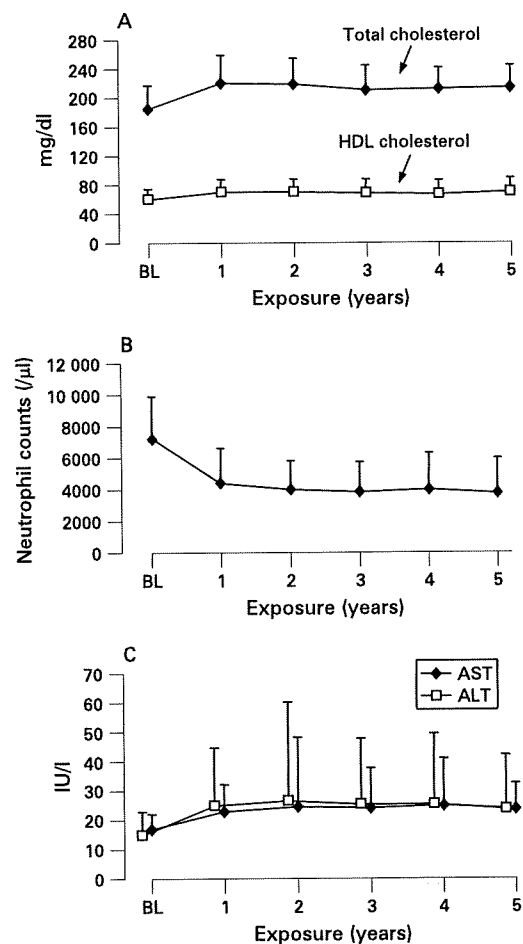


Figure 2 Change in serum total cholesterol, high-density lipoprotein (HDL) cholesterol, neutrophil counts, aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Values are means. Bars indicate SD. BL, baseline.

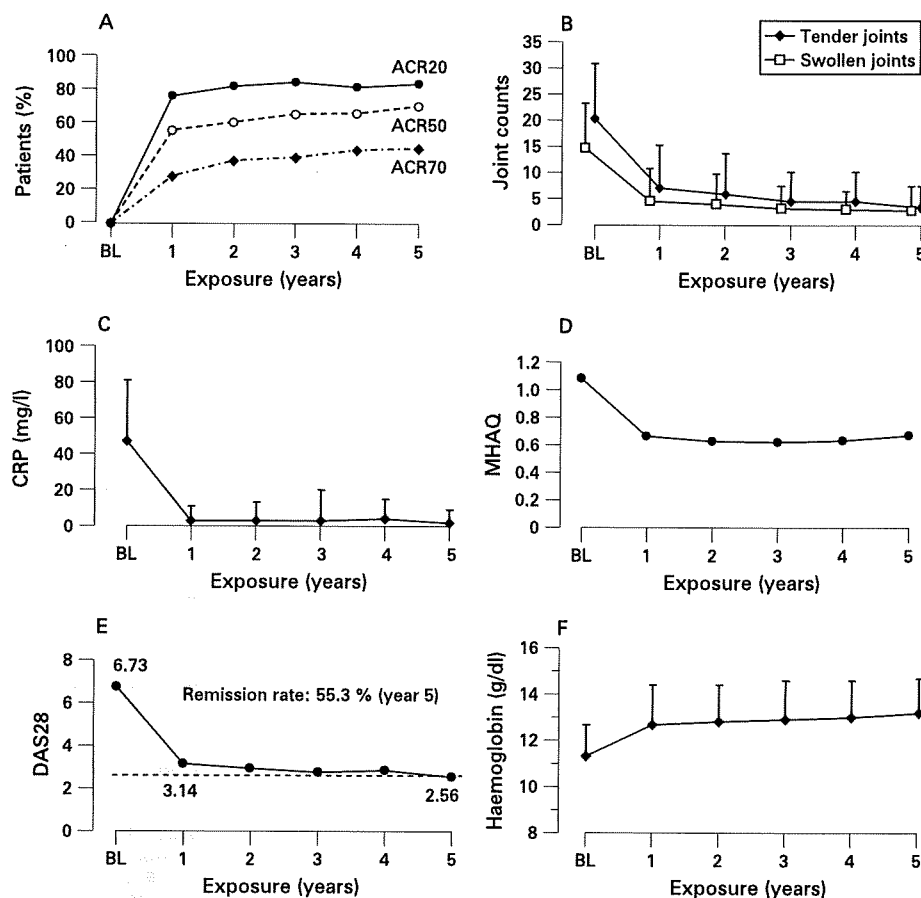
respectively, during the study, but most were transient and resolved without any particular treatment. No serious liver disorders, such as fulminant hepatitis, were seen during this study.

Efficacy

The response rate according to the ACR improvement criteria increased during the initial year and remained constant throughout the study period (fig 3A). At 5 years, 79 (84.0%), 65 (69.1%) and 41 (43.6%) of 94 patients met ACR20, ACR50 and ACR70, respectively. These response rates analysed with the last observation carried forward were 77.3%, 58.9% and 37.6%, respectively.

Tocilizumab treatment significantly improved all measures, including tender joint counts, swollen joint counts (fig 3B), CRP levels (fig 3C), MHAQ score (fig 3D) and DAS28 score (fig 3E), and the efficacy was sustained throughout the 5-year treatment. The percentage of patients who achieved clinical remission defined as DAS28 less than 2.6^{13 14} was 55.3% (52/94) at 5 years. Most patients exhibited anaemia at baseline and the mean haemoglobin level was 11.3 mg/dl (SD 1.4). Tocilizumab treatment significantly improved anaemia in these patients, and the mean haemoglobin level was increased to 13.2 mg/dl (SD 1.5) at year 5 (fig 3F).

Figure 3 Percentage of responders according to the American College of Rheumatology improvement criteria and the disease activity score in 28 joints (DAS28) as well as the mean change in modified health assessment questionnaire (MHAQ) scores, number of tender joints, number of swollen joints, C-reactive protein (CRP) and haemoglobin. BL, baseline.



Eighty-eight of the 94 patients who received tocilizumab for more than 5 years had received corticosteroids when they began the initial study. After 5 years of tocilizumab treatment, 78 of 88 (88.6%) had been able to decrease their corticosteroid dose and 28 of 88 (31.8%) had discontinued corticosteroids. The mean dose of corticosteroids for these patients decreased from 6.9 mg/day (median 7.5 mg/day) to 2.4 mg/day (median 2.0 mg/day) at 5 years.

DISCUSSION

The STREAM study is the first study demonstrating the long-term safety and efficacy of tocilizumab monotherapy. This open-label extension trial of tocilizumab demonstrated a sustained good efficacy and a generally good safety profile over 5 years. The high retention rate at 5 years indeed indicates the favourable efficacy and safety profile. In particular, only one of 143 patients withdrew as a result of an unsatisfactory response, indicating that no general loss of response occurred during long-term treatment.

ACR responses and improvements in DAS28 scores and individual components of the ACR core set were all sustained during the long-term treatment with tocilizumab monotherapy. At 5 years, approximately half of patients had achieved ACR70 and more than half of patients had achieved clinical remission defined as a DAS28 of less than 2.6, although this study was open labelled.

Tocilizumab monotherapy markedly improved inflammation markers such as CRP and ESR and improvements were sustained throughout the study. Haemoglobin levels were also improved. It is well documented that hepcidin plays a key role in anaemia of chronic inflammatory diseases. IL-6 induces the secretion of hepcidin, an iron regulatory peptide hormone that is produced in the liver and that negatively regulates the

absorption of intestinal iron and iron recycling by macrophages.¹⁵ This increase in haemoglobin levels is expected to contribute to the improvement in patients' quality of life.

A steroid-sparing effect was another benefit of tocilizumab therapy for RA patients. As the use of corticosteroids is often associated with adverse events such as infection or steroid-induced osteoporosis, this also contributes to the improvement in patients' quality of life from the safety point of view.

A major objective of this study was to evaluate long-term safety. Long-term treatment with tocilizumab was well tolerated. Most of the adverse events were mild and acceptable compared with the benefit provided. The rate of serious infections of 5.7/100 patient-years after 612 patient-years of treatment was comparable to that reported with tumour necrosis factor (TNF) antagonists.^{16, 17} There was no systemic opportunistic infection or tuberculosis in this study. At least two patients with a history of tuberculosis were treated with tocilizumab because this study did not exclude patients who had a history of tuberculosis. Neither had any recurrence nor exacerbation of tuberculosis without the prophylactic use of antituberculosis drugs. However, two cases of tuberculosis were reported in another study (two cases in 1891 patient-years in Japan),¹⁸ and we should therefore follow patients carefully during tocilizumab treatment.

Four malignancies were reported in four patients. Yamanaka *et al*¹⁹ reported a comparison of the incidence of malignancies in the following three populations: (1) tocilizumab cohort: all clinical trials (including this trial) of tocilizumab in active RA patients; (2) IORRA cohort: an observational cohort of RA patients in the Institute of Rheumatology, Tokyo Women's Medical University and (3) a Japanese population database: cancer incidence in Japan

Extended report

by the research group for population-based cancer registration in Japan supported by the Japanese Ministry of Health, Labour and Welfare. The incidence of malignancies in the patients receiving tocilizumab was almost equivalent to that in the observational cohort of RA patients or the Japanese population data. Further study will be required to evaluate whether tocilizumab treatment might influence the incidence of malignancies using a much larger population of RA patients treated with tocilizumab.

Throughout long-term treatment, a serious infusion reaction was observed in only one patient who received 4 mg/kg tocilizumab in the initial double-blind trial and developed IgE anti-tocilizumab antibodies. Maini *et al*⁶ reported that anaphylaxis and anaphylactoid reactions occurred only at low doses of tocilizumab in the absence of methotrexate. Therefore, initial treatment with a relatively low dose (4 mg/kg) of tocilizumab without methotrexate may induce anti-tocilizumab antibodies.

Increases in total cholesterol, high-density lipoprotein cholesterol and triglycerides were observed in the initial controlled study. In this extension study, however, they did not continue increasing. Furthermore, the atherogenic index, calculated by (total cholesterol-high-density lipoprotein cholesterol)/high-density lipoprotein cholesterol, was stable throughout the 5-year treatment. Therefore, an increase in total cholesterol does not always mean an increased risk of cardiovascular disease. As IL-6 is thought to play a causative role in atherosclerosis, IL-6 blockade may decrease the incidence of cardiovascular events, as observed with anti-TNF therapy.²⁰ Further investigation will be required to evaluate whether tocilizumab might increase the risk of developing ischaemic heart disease. At present, we should introduce treatment according to the guideline for cholesterol management.

Neutropenia was also reported, as seen in previous studies,^{4-7, 9} but the incidence was less frequent than that observed in combination with methotrexate therapy.^{6, 7, 9} This may be an advantage of tocilizumab monotherapy.

Although it has been established that TNF inhibitors should be given with methotrexate for maximal efficacy,^{21, 22} this study indicated that tocilizumab monotherapy offered a good safety profile and sustained efficacy throughout long-term treatment. Therefore, tocilizumab has considerable clinical benefit for patients who do not tolerate methotrexate. Short-term safety and efficacy studies of tocilizumab in combination with methotrexate or DMARD have been reported,⁶⁻⁹ but further studies are required to determine long-term safety and efficacy.

In conclusion, this study clearly demonstrates excellent long-term efficacy and generally good safety of tocilizumab monotherapy in active RA patients.

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Competing interests: NN has served as a consultant to and received honoraria from Chugai Pharmaceuticals, the manufacturer of tocilizumab. NN also works as a scientific advisory board of Hoffmann-La Roche who develops tocilizumab in collaboration with Chugai Pharmaceutical Co Ltd. The other authors have no competing interests.

Ethics approval: Ethics approval was obtained.

Patient consent: Obtained.

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Tacrolimus therapy for systemic lupus erythematosus without renal involvement: a preliminary retrospective study

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Abstract We conducted a pilot study to investigate whether tacrolimus was effective for treating patients with systemic lupus erythematosus (SLE) without renal involvement. Ten SLE patients with symptoms such as arthritis and erythema, but no active nephritis, were treated with tacrolimus. They included 8 women and 2 men aged from 24 to 62 years [mean \pm standard deviation (SD): 42.1 ± 11.3 years]. Tacrolimus was administered at doses of 1–3 mg daily, and efficacy was assessed from the SLE Disease Activity Index (SLEDAI) after 1 year. Two patients ceased treatment due to adverse reactions (after 4 days for chest pain and 7 months for recurrent infections). The other 8 patients completed 1 year of treatment, and significant improvement of disease activity was observed in 6 of them. The mean (\pm SD) SLEDAI showed a significant decrease after 1 year of tacrolimus therapy, from 6.8 ± 3.1 to 3.4 ± 0.9 ; $p < 0.05$ by Student's paired t test. The mean (\pm SD) dose of prednisolone also decreased significantly, from 16.8 ± 8.6 to 9.3 ± 4.6 mg/day; $p < 0.05$. Although a prospective controlled study will be necessary to confirm, tacrolimus might be a treatment option for active SLE without renal involvement.

Keywords Systemic lupus erythematosus · Tacrolimus · Retrospective study · SLEDAI

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that causes various symptoms and immunological abnormalities. The standard treatment is corticosteroid monotherapy, but a combination of corticosteroid with other immunosuppressive agents, such as cyclophosphamide, azathioprine, or mycophenolate mofetil, is used for SLE with severe organ involvement [1].

Tacrolimus is an immunosuppressant derived from *Streptomyces tsukubaensis* that was discovered by Kino et al. in 1984 [2]. At first, tacrolimus was used to suppress rejection after organ transplantation, and it was shown to improve the survival rate [3]. More recently, tacrolimus has also been successfully applied for the treatment of rheumatoid arthritis [4]. Furthermore, it has been reported that tacrolimus is effective for various other autoimmune diseases, inflammatory bowel disease, myasthenia gravis [5–8], and lupus nephritis [9, 10]. In Japan, a placebo-controlled clinical trial of tacrolimus for lupus nephritis was performed to investigate the efficacy and safety of this agent. Based on the results obtained, administration of tacrolimus at an oral dose of 3 mg/day was approved for the treatment of lupus nephritis in Japan in 2007. However, reports regarding the use of tacrolimus for SLE patients without renal involvement are limited. Accordingly, this study was performed to clarify the efficacy of tacrolimus in such patients.

Methods

Patients

This retrospective cohort study was approved by the Ethical Committee of Toho University School of Medicine.

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Table 1 Baseline characteristics of the 10 systemic lupus erythematosus (SLE) patients

Patient no.	Age/sex	Duration (years)	Clinical features	Low complement	Anti-DNA antibody (IU/ml)	PSL (mg/day)	SLEDAI score
1 ^a	42/F	26	Ar, C	Yes ^e	ND	15	12
2	62/F	3.5	Ar	Yes	ND	12	6
3	45/F	16	Ar	No	ND	10	6
4	34/F	1.5	Ar	Yes	ND	35	6
5 ^b	58/F	3.3	Ar, M, Al, C, O, L	Yes	ND	24	13
6	49/F	26	Ar	Yes	19	10	8
7	41/F	27	T	Yes	ND	15	3
8	30/M	5	C, Ly	No	14	13	4
9 ^c	24/M	9	C	Yes	97	14	8
10 ^c	38/F	14	Al	Yes	14	10	4
	42 ± 11 ^d	13 ± 10				15.8 ± 8.5	6.6 ± 2.7

PSL prednisolone, SLEDAI SLE Disease Activity Index, Ar arthritis, C cutaneous manifestations, M myositis, Al alopecia, O oral ulcer, L leukopenia, T thrombocytopenia, Ly lymphopenia, ND not detected

^a Received methotrexate therapy

^b Overlap of SLE and dermatomyositis

^c Tacrolimus was discontinued due to an adverse event

^d Mean ± SD

^e Lower value from respective normal range at least one of the serum C3, C4 and/or CH50 measures

Prior to the study, written informed consent was obtained from all patients regarding the use of information from their medical records for research purposes. The subjects included 10 SLE patients who started tacrolimus therapy at Toho University School of Medicine between April 2005 and February 2006 (Table 1). All patients were diagnosed as having SLE according to the 1997 revision of the American College of Rheumatology criteria [11]. The inclusion criteria for the study were adult patients with active symptoms for at least 3 months despite receiving prednisolone at daily doses of 10 mg or more. The patients had various symptoms, such as arthritis, erythema, myositis, alopecia, oral ulcers, leukopenia, lymphopenia, and thrombocytopenia. However, evidence of active nephritis was not detected by laboratory tests: serum creatinine was <0.7 mg/dl; proteinuria was <0.5 g/day (or less than 3+), and cellular casts were not detected on urinalysis. No patient had severe organ involvement, such as central nervous system disorders or interstitial lung disease.

Tacrolimus treatment and study protocol

As shown in Table 2, tacrolimus was administered at doses between 1 and 3 mg daily (mean initial dosage: 2.5 ± 0.9 mg/day), being given once or twice daily after meals. Doses of tacrolimus were given once after the evening meal at 1 year. Other immunosuppressants, such as azathioprine, mycophenolate mofetil, and cyclosporine, were not used from at least 3 months before enrollment in

Table 2 Doses and blood levels of tacrolimus in the 10 systemic lupus erythematosus (SLE) patients

Patient no.	Initial dose of tacrolimus (mg/day)	Dose of tacrolimus after 1 year (mg/day)	Blood level of tacrolimus after 1 year (ng/ml)
1	1	3	6.4
2	3	2	3.7
3	3	3	7.9
4	1.5	2	4.0
5	3	2.5	4.1
6	3	3	8.2
7	3	3	10.7
8	3	3	6.1
9	3	–	–
10	1	–	–
	2.5 ± 0.9	2.7 ± 0.5	6.4 ± 2.5

the study or during the study period. The dose of prednisolone could be altered depending on the level of disease activity. Patients were followed at 1-month intervals for 1 year, with the physical findings, serum chemistry, urinalysis, and blood cell count being monitored.

Parameters and statistics

The SLE Disease Activity Index (SLEDAI) was determined as reported elsewhere [12], and the change of the SLEDAI score was the primary end-point of this study. In

addition, changes in the serum levels of C3 and immunoglobulin G (IgG) were assessed. Comparison of these values before and after 1 year of tacrolimus treatment was done by Student's paired *t* test, and a *P* value of less than 0.05 was considered to indicate statistical significance.

Results

Patient profile

Table 1 presents the clinical profile of the 10 SLE patients, who included 8 women and 2 men with a mean age of 42 years at the time of enrollment. Most patients had a long history of SLE (mean duration 13 years). Four of the 10 patients (1, 2, 4, and 5) had previously received azathioprine, which had been discontinued due to adverse reactions in patients 1 and 2 and due to lack of efficacy in patients 4 and 5. Patient 2 had also received cyclosporine, which was discontinued because it was not effective. Patient 5 had used methotrexate but discontinued treatment due to adverse reactions. Patient 1 was on weekly methotrexate pulse therapy (7.5 mg/dose) at the start of the study, whereas the other patients were not receiving any

immunosuppressive agents apart from prednisolone at the doses shown in Table 1. After the start of tacrolimus administration, 2 patients were withdrawn from the study due to adverse reactions, whereas the other 8 patients continued treatment for more than 1 year.

Effect of tacrolimus on the SLEDAI score

Figure 1 presents changes of SLEDAI scores from baseline in the 8 patients who continued treatment with tacrolimus for 1 year. Compared with the pretreatment score, there was a significant decrease after 1 year. When individual changes were assessed, 6 patients showed a decrease in the score. In addition, there was a significant reduction in the dose of prednisolone after 1 year of treatment compared with baseline in all 8 patients (Fig. 2).

Clinical efficacy of tacrolimus

Arthralgia was present at the start of treatment in 6 patients and resolved within 12 weeks in all of them. Patient 5 had leucopenia, erythema, myositis, and alopecia besides arthritis, but all of these symptoms improved after 1 month of treatment with tacrolimus (Table 1). In patient 7, no

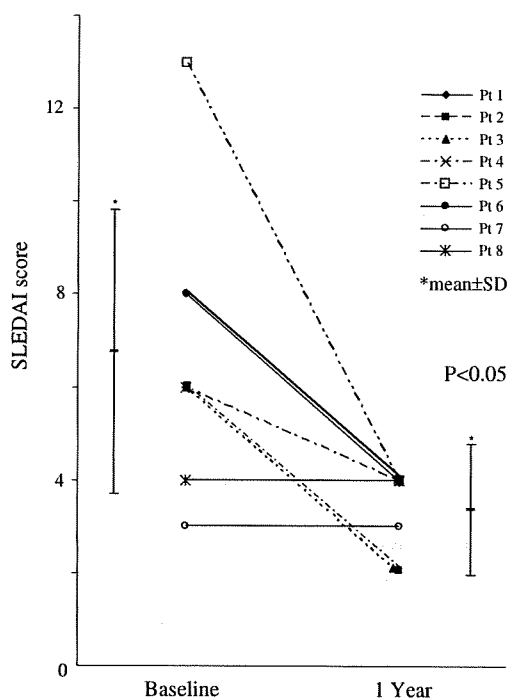


Fig. 1 Change of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score after tacrolimus therapy in 8 SLE patients. The mean [\pm standard deviation (SD)] score was 6.8 ± 3.1 before tacrolimus treatment and decreased significantly ($p < 0.05$) to 3.4 ± 0.9 after 1 year

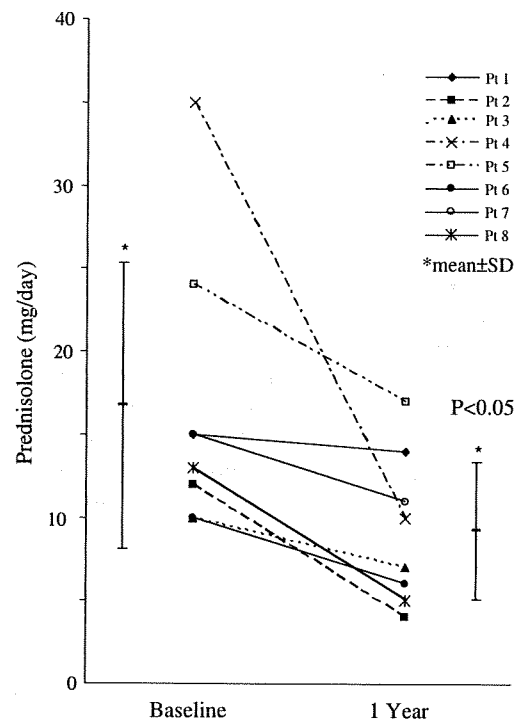


Fig. 2 Reduction of prednisolone dose during tacrolimus therapy in 8 systemic lupus erythematosus (SLE) patients. The mean [\pm SD] dose of prednisolone was 16.8 ± 8.6 mg/day before tacrolimus treatment and decreased significantly ($p < 0.05$) to 9.3 ± 4.6 mg/day after 1 year

Fig. 3 Changes of serum immunoglobulin G (IgG) during tacrolimus treatment in 8 systemic lupus erythematosus (SLE) patients. The mean (\pm SD) serum IgG level decreased from $1,452 \pm 371$ to $1,205 \pm 277$ mg/dl after 1 year

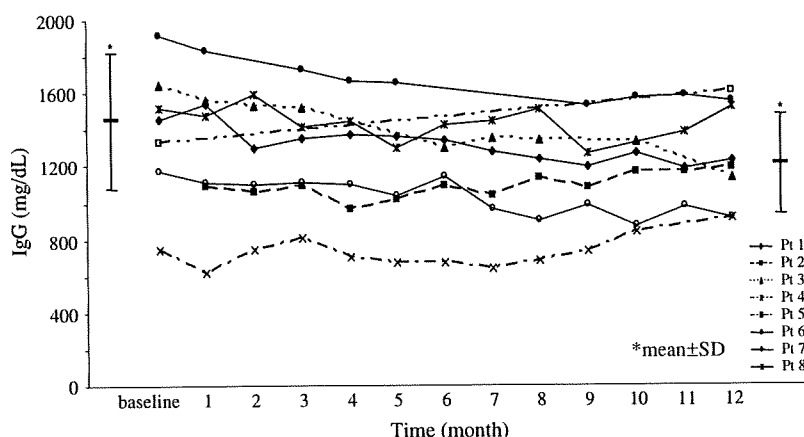
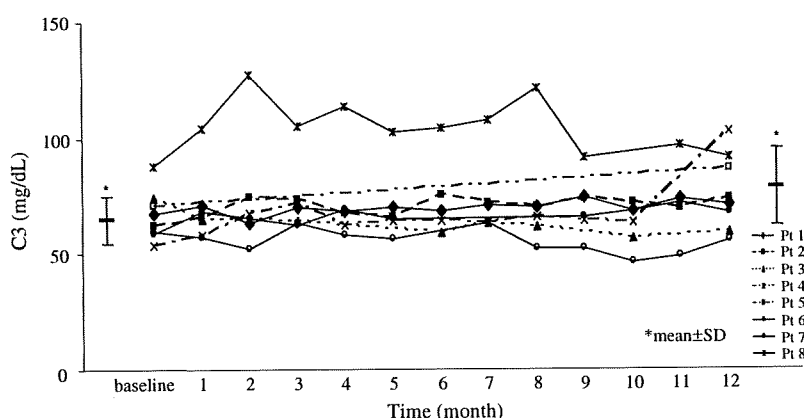


Fig. 4 Changes of serum C3 during tacrolimus treatment in 8 systemic lupus erythematosus (SLE) patients. The mean (\pm SD) serum C3 level increased from 64.5 ± 10.8 to 72.0 ± 16.2 mg/dl after 1 year. The normal range of C3 is from 64 to 116 mg/dl in our hospital



improvement of thrombocytopenia was observed after the start of tacrolimus therapy, and there was also no improvement of the SLEDAI score. Patient 8 showed no improvement of cutaneous manifestations, but lymphopenia was improved.

Changes of serum IgG after the start of tacrolimus treatment are presented in Fig. 3. The mean serum IgG concentration was 1,452 mg/dl immediately before the start of tacrolimus administration and showed a decrease to 1,205 mg/dl after 1 year, but there was no significant statistical difference ($P = 0.14$). Figure 4 shows the changes of serum C3 after the start of treatment with tacrolimus. The mean serum C3 concentration was 64.5 mg/dl immediately before starting tacrolimus and increased to 72.0 mg/dl after 1 year, but again, there was no significant statistical difference ($P = 0.36$). The titers of anti-DNA antibodies (measured by radioimmunoassay) were positive in 4 patients (19, 14, 97, and 14 IU/ml in patients 6, 8, 9, and 10, respectively). Patients 9 and 10 were withdrawn at 4 days and 7 months after tacrolimus treatment, respectively. Anti-DNA antibody titers in patients 6 and 8 were changed from 19 IU/ml and 14 to 9 and 11 IU/ml, respectively.

Table 3 Adverse reactions to tacrolimus

Patient no.	Adverse event	Outcome
2	Finger tremor	Improved after dose reduction
5	Headache	Improved after dose reduction
9	Chest pain	Withdrawn after 4 days
10	Recurrent infections	Withdrawn after 7 months

Adverse events

Table 3 presents a summary of the adverse events that occurred during administration of tacrolimus. Four of the 10 patients suffered from adverse events, and 2 patients discontinued treatment. Patient 2 noted tremor of the fingers beginning several days after starting treatment with tacrolimus, but the symptom improved when the dose was reduced from 3 to 2 mg/day. Patient 5 developed a headache when tacrolimus treatment was started, but this symptom resolved after dose reduction from 3 to 2 mg. Patient 9 was withdrawn from the study after 4 days of treatment due to chest pain, whereas patient 10 was withdrawn from the study due to recurrent folliculitis after

7 months of treatment. In all 4 patients, symptoms resolved after dose reduction or discontinuation of tacrolimus, and no sequelae were observed.

Discussion

The results of this retrospective study suggest that oral administration of tacrolimus is safe and effective for SLE patients without serious organopathy. Although the subjects of this study were not in a poor general condition, they had not been able to reduce the dose of prednisolone to <10 mg/day for at least 3 months due to the symptoms listed in Table 2. We often encounter SLE patients without serious organ involvement, such as nephropathy or central nervous system lesions, who still need to take a relatively high dose of corticosteroids because of persistent disease activity. Even at low to medium doses, corticosteroids are known to have an unfavorable influence on the quality of life and prognosis of patients with rheumatoid arthritis [13], so low to medium doses of corticosteroids are expected to have a negative influence in SLE patients. Therefore, immunosuppressive agents are coadministered in order to reduce the corticosteroid dose.

Dudridge and Powell [14] administered tacrolimus to 3 SLE patients and found that cutaneous vasculitis, leukopenia, arthritis, and hypocomplementemia improved in 2 patients. In 2006, Maruoka et al. [15] reported that tacrolimus was effective in a patient with lupus cystitis. However, there has been no previous assessment of long-term tacrolimus therapy in a larger group of SLE patients without nephropathy, as was done in this study. We demonstrate that treatment with tacrolimus for 1 year could maintain suppression of disease activity in SLE patients without renal involvement so that reduction of the corticosteroid dose was possible.

There have been a few studies on the response to other immunosuppressive agents in SLE patients with mild symptoms. For example, azathioprine was reported to be effective for cutaneous lupus erythematosus [16]. However, according to another report, azathioprine was not effective for arthritis, serositis, or cutaneous manifestations of SLE [17]. Mok [18] conducted a systematic review of the efficacy of mycophenolate mofetil for SLE without renal involvement and concluded that it could improve hematological and dermatological symptoms. A number of reports on methotrexate have been published, and the findings were summarized by Wong et al. [19]. Data from an uncontrolled case series of 20 patients with and without nephropathy and one retrospective cohort study suggested that methotrexate is probably effective against active joint disease and skin manifestations. Two prospective randomized trials showed a good response, but another did not

demonstrate any benefit of methotrexate. Treatment of SLE with cyclosporine, which has the same mechanism of action as tacrolimus (inhibition of calcineurin activity), has also been reported. Caccavo et al. [20] found that combined cyclosporine and corticosteroid therapy was effective for skin symptoms, arthritis, and serositis in 30 SLE patients, but a number of adverse drug reactions occurred. There have been other reports about that effectiveness of cyclosporine for mild symptoms of SLE [21–25], but the incidence of adverse drug reactions seems to be high (40–60%).

Clinical trials of patients with rheumatoid arthritis have shown that the main adverse reactions caused by tacrolimus are glucose intolerance, renal impairment, hypertension, and gastrointestinal symptoms [4]. In our study, adverse reactions were observed in 4 patients, but all symptoms were reversible and improved after dose reduction or discontinuation of tacrolimus. We [26] previously studied the safety profile of tacrolimus and found that 21 out of 42 patients discontinued treatment due to adverse reactions during an average observation period of 288 days. In the study presented here on patients with SLE, no gastrointestinal symptoms were observed, although these were the most common adverse reaction (45.2%, 19/42 patients) [26] in patients with rheumatoid arthritis. The reason for this difference between patients with SLE and rheumatoid arthritis is not known, but the different underlying diseases, patient's age, and concurrent treatment (e.g., nonsteroidal anti-inflammatory drugs) may have played a role.

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Conflict of interest statement Dr. Shinichi Kawai has served as a consultant to and/or received research grants and honoraria from Astellas Pharma Inc. (Tokyo, Japan), the manufacturer of tacrolimus. The other authors declare no conflict of interest.

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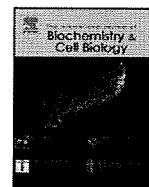
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Real-time functional imaging for monitoring miR-133 during myogenic differentiation

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ABSTRACT

MicroRNAs (miRNAs) are a class of non-coding small RNAs that act as negative regulators of gene expression through sequence-specific interactions with the 3' untranslated regions (UTRs) of target mRNA and play various biological roles. miR-133 was identified as a muscle-specific miRNA that enhanced the proliferation of myoblasts during myogenic differentiation, although its activity in myogenesis has not been fully characterized. Here, we developed a novel retroviral vector system for monitoring muscle-specific miRNA in living cells by using a green fluorescent protein (GFP) that is connected to the target sequence of miR-133 via the UTR and a red fluorescent protein for normalization. We demonstrated that the functional promotion of miR-133 during myogenesis is visualized by the reduction of GFP carrying the miR-133 target sequence, suggesting that miR-133 specifically down-regulates its targets during myogenesis in accordance with its expression. Our cell-based miRNA functional assay monitoring miR-133 activity should be a useful tool in elucidating the role of miRNAs in various biological events.

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1. Introduction

MicroRNAs (miRNAs) are a class of non-coding small RNAs that act as negative regulators of gene expression by promoting mRNA degradation and/or repressing translation through sequence-specific interactions with the 3' untranslated regions (UTRs) of the target mRNA (Bartel, 2004; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Hundreds of miRNAs have been found in various organisms, and many miRNAs are evolutionarily conserved, suggesting their important roles in biological processes (Bartel, 2004). Some miRNAs show localized expression patterns with limited tissue, cell, and spatiotemporal specificities. Moreover, one-third of all mammalian mRNAs seem to be under miRNA regulation (Lewis et al., 2005). Therefore, miRNAs play an essential role in regulating gene expression.

Recent reports using microarray analyses with bioinformatics have identified miR-1, miR-133, and miR-206 as muscle-specific miRNAs that regulate muscle growth and differentiation (Brennecke et al., 2005; Chen et al., 2006; Rao et al., 2006). Both miR-1 and miR-133 are evolutionarily conserved since they have been found in most animal species, from *Drosophila* to

humans. The miR-133 levels increase during the course of myogenic differentiation. However, miR-133 has been reported to enhance myoblast proliferation, despite the fact that miR-1 and miR-206 promote muscle differentiation (Chen et al., 2006). To understand the molecular network involving miRNAs in myogenesis, it is crucial to monitor the dynamics of miRNAs during myogenesis.

To determine the expression levels of miRNAs, Northern blotting and RT-PCR with microarray analysis are often carried out for the direct detection of miRNA. For miRNA visualization, *in situ* hybridization analysis is conventionally performed using specific probes and fixed tissues. Indirect detection of miRNAs entails the use of reporter genes whose UTRs are connected with the target sequence of the miRNAs (Brown et al., 2007, 2006; Mansfield et al., 2004; Zeng et al., 2002). In this system, if the target sequence of miRNAs is located downstream of the reporter genes, including β -galactosidase or luciferase, miRNAs induce a decrease in reporter signaling by reducing protein translation (Mansfield et al., 2004; Zeng et al., 2002). However, there are few reports on monitoring the dynamic function of miRNAs in intact cells or organs among the mixture of closely associated cell state. Recently, Naldini et al. reported that a lentiviral vector encoding green fluorescent protein (GFP) connected to a target sequence allowed them to visualize the activity of miR-142-3p followed by immunostaining of an internal control gene using fixed tissues (Brown et al., 2006, 2007). In this study, we developed a novel retroviral vector to monitor

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the specific miRNA activity in living cells. Using two fluorescent proteins as reporters, the miRNA activity in living cells can be directly analyzed using fluorescence microscopy. Our functional analysis using a retroviral vector is a useful method to examine the dynamic activity of miRNA in living cells.

2. Materials and methods

2.1. Construction of the retroviral vector

The fragment encoding GFP fused with the blasticidin-resistant gene was amplified by PCR by using the primers 5'-AGGGATC-CGCCACCATGGTGAGCAAGGGCGAG-3', 5'-ACTACTCGAGGTTAAC-GAATTCTAGCCCTCCAC-3', and 5'-GACAAAGGCTTGGCCTGGCCAT-CGATTTTGTACAGCTCGTCCATGC-3' with pEGFP-C3 and pTracer-EF/bsd as the templates. The resultant product digested with BamHI/XhoI was integrated into pMX-puro to yield pMXGb. The fragment carrying RFP was amplified by PCR with the primers 5'-CGGAAGCTTGCCACCATGGTGAGCAAGGGCGA-3' and 5'-AAAAGTCCGACTAATCGATCTTGTACAGCTCGTCCATGCCG-3' by using pRSET-mCherry (Shaner et al., 2004) as a template. The resultant product digested with HindIII/SalI was inserted into pMXGb to yield pMXRgB. The CMV promoter was amplified by PCR with the primers 5'-AACTCGAGTAGTTATAATAGTAATCAATTACGG-3' and 5'-ACAAGCTTCTAGTGATCTGACGGTTCCTAA-3', using pEGFP-C3 as a template. The resultant product digested with XhoI/HindIII was inserted into pMXRgB to yield pMXCRgB.

The fragment corresponding to a three tandem repeat of the target sequence that was completely complementary to miR-133 was prepared by annealing two oligonucleotides: 5'-AATTACAGC-TGGTTGAAGGGGACCAACAGCTGGTTGAAGGGGACCAACAGCTGGTT-GAAGGGGACCAA-3' and 5'-TCGATTGGTCCCTTCAACCAGCTGTTG-TCCCTTCAACCAGCTGTTGGTCCCTTCAACCAGCTGT-3'. The resultant fragment was inserted into pMXCRgB at the EcoRI/XhoI sites to yield pMXCRgB[133].

2.2. Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed according to the previous report (Wilkinson, 1992) with minor modifications. In brief, the embryos (E11.5) were fixed with 4% PFA, 0.2% glutaraldehyde for 20 min at room temperature. Digoxigenin (DIG)-labeled antisense probes (~500 ng/ml) were hybridized for over 14 h at 70°C. The embryos were treated with an anti-DIG AP Fab fragment antibody (Roche, Mannheim, Germany) with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Roche). A mouse primary miR-133a2 fragment was amplified from mouse cDNA by PCR with the primers 5'-TCTGCCTTCCAGCCATG-3' and 5'-GATCCACTGGGAGGAGACTCC-3'. Since the mature region was too short to detect miR-133 under our condition, we used a probe against primary region of miR-133a, although LNA instead of RNA probes are more appropriate for mature miRNA detection (Wienholds et al., 2005). DIG-labeled probes were transcribed with a DIG-RNA labeling kit and T7 RNA polymerase (Roche).

2.3. Induction of myogenesis

Mouse myoblast C2C12 cells were cultured in growth medium (GM) comprising DMEM (Dulbecco's modified Eagle's medium; Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum. They were cultured until they reached a high-confluent density, and the medium was changed to differentiation medium (DM) comprising DMEM supplemented with 2% horse serum to induce differentiation.

2.4. Culture and infection of cells

Retrovirus packaging cell line Plat-E (kindly provided by T. Kitamura) cells were cultured in DMEM supplemented with 10% fetal bovine serum in the presence of 1.0 µg/ml puromycin and 10 µg/ml blasticidin (Morita et al., 2000). The cells were transfected with a transfection reagent, TransIT-293 (Pan Vera, Madison, WI), according to the manufacturer's protocol. The cells were cultured to a confluence of approximately 80% (1×10^7 cells) and transfected with the retroviral vector. After 18 h, the culture medium was changed to fresh DMEM and further incubated for 30 h, at which point the culture medium was collected and passed through a 0.45-µm filter. C2C12 myoblasts were transduced with the retrovirus from the filtrated medium plus 5 µg/ml polybrene (Sigma). Two days after infection, the cells were selected by 10 µg/ml blasticidin (Sigma). Imaging of transduced cells was done by microscope Eclipse (Nikon, Tokyo, Japan) and 10–20 clones were screened.

2.5. Northern blotting

Total RNA was extracted and purified with the ISOGEN reagent (Wako, Osaka, Japan). An aliquot of total RNA (10 µg per lane) was loaded on a 12% polyacrylamide denaturing gel. After electrophoresis, bands of RNA were electro-transferred to a Hybond-XL membrane (Amersham Biosciences, Piscataway, NJ). The membrane was probed with ³²P-labeled RNA that was complementary to the individual miRNA sequence (Sano et al., 2006).

2.6. Flow cytometry analysis

Synthetic double-stranded RNA (dsRNA) of miR-133 (5'-GCUGGUAUAAUGGAACCAAU-3' and 5'-UUUGUCCCUU-CAACCAGCUG-3') was transfected with C2C12 myoblasts using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 2 days after transfection of miR-133, 10,000 cells from each samples were trypsinized and analyzed using a FACS Aria (Becton Dickinson, Sparks, MD). The fluorescent signals from GFP or RFP excited by a laser at 488 nm were monitored by their emissions using 515–545 nm (FL1) or 675–715 nm (FL3) filter, respectively.

3. Results

3.1. Expression of miR-133 during muscle development

The mouse skeletal muscle myogenic progenitor C2C12 cell line possesses pluripotent differentiation potential in mesenchymal cell lineages *in vitro* (Blau et al., 1985; Soulez et al., 1996). The expression of miR-133 is reported to increase during the course of myogenic differentiation of C2C12 myoblasts (Chen et al., 2006). Our Northern blotting analysis revealed that expression of miR-133 was increased approximately 6-fold at 4–7 days after changing the culture medium for C2C12 from GM to DM; this is in agreement with the results of other groups (Chen et al., 2006; Kim et al., 2006; Liu et al., 2007; McCarthy, 2008; McCarthy et al., 2007; Rao et al., 2006) (Fig. 1A). Quantitative RT-PCR analysis revealed that the miR-133 expression during myogenesis increased approximately 5-fold (Fig. 1B). miR-133 correlated with myogenic differentiation leads to a potential marker molecule for myogenic differentiation, similar to myogenin and myoD, both of which are upregulated in response to myogenic differentiation (Arnold and Braun, 1996). Next, we attempted a whole-mount *in situ* hybridization of primary miR-133 (pri-miR-133) to evaluate the developmental role of miR-133. pri-miR-133 was clearly observed in somites in the developing mice (Fig. 1C, visualized as violet); however, *in situ* hybridization with fixed and permeabilized tis-

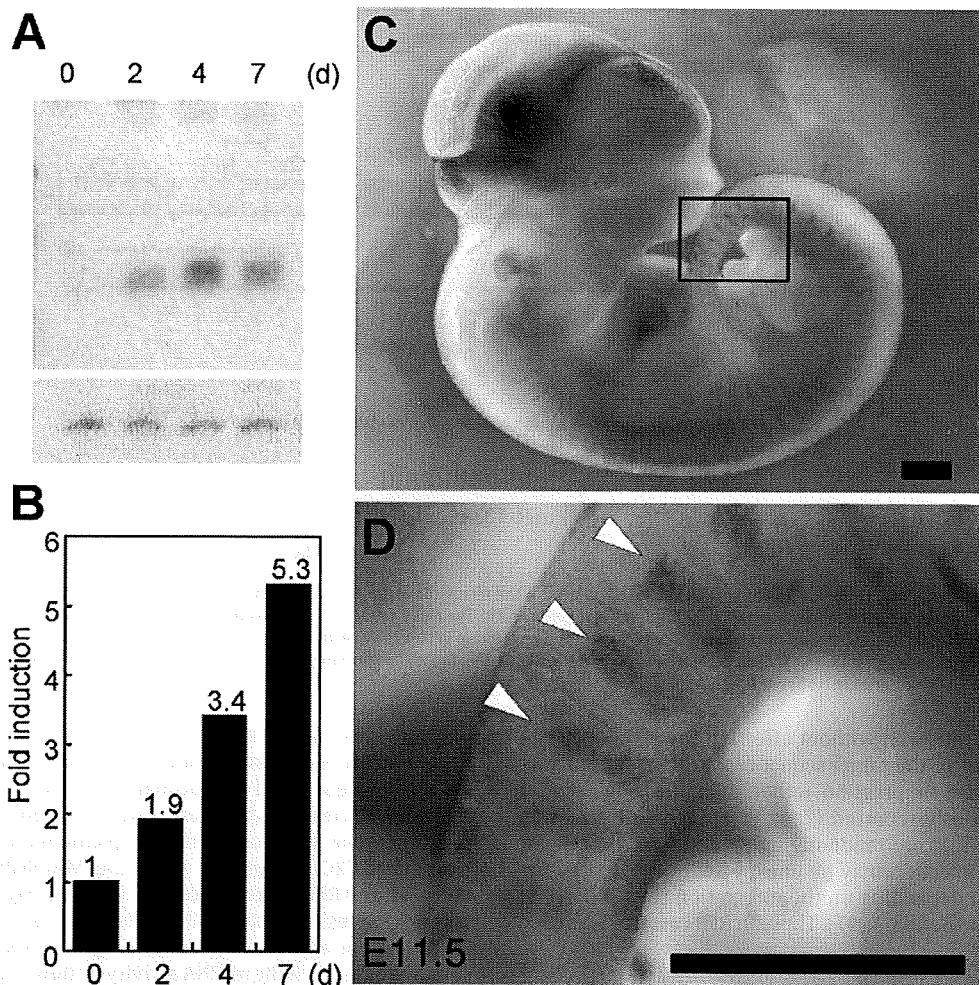


Fig. 1. Expression patterns of miR-133. (A) Northern blots showing the kinetics of miR-133 expression at various time periods (0, 2, 4, and 7 days) after changing from GM to DM during the myogenesis of C2C12 myoblasts. 5S rRNA was used as a loading control. (B) Expression of miR-133 was determined by quantitative RT-PCR analysis. Results are means of results from three experiments. (C) and (D) Following the fixation of mouse embryos, whole-mount *in situ* hybridization was performed using a DIG-labeled probe against primary miR-133 (violet, allowheads). (Scale bar: 50 μ m) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sues were not suitable for determination of the specific period of cells that are responsible for the expression of miR-133 in the early myogenesis.

3.2. Structure of the retroviral vector encoding green and red fluorescent proteins

To monitor myogenic differentiation in living cells, we constructed a dual-color monitoring system by using a retroviral vector that encodes two different fluorescent proteins. The retroviral vector is based on the Moloney murine leukemia retroviral (M-MLV) vector pMX (Morita et al., 2000), as shown in Fig. 2. Since our retroviral vector encodes two long terminal repeats (LTRs) whose promoters are both active but do not show self-inactivation (SIN), upstream transcription occurs after the integration of the retrovirus in the cellular genome. The provirus yields two independent transcripts: one mRNA encodes the red fluorescent protein (RFP) under the control of the cytomegalovirus (CMV) promoter (transcript 1, Fig. 2B) and the other mRNA encodes GFP under the control of the LTR promoter (transcript 2, Fig. 2B). Transcript 2 possesses the sequences for both RFP and GFP; however, RFP is not translated into protein because eukaryotes do not allow translation of the second open reading frame (ORF) from the 5' cap terminus. The

R region of 3' LTR is used for a common poly-adenylation signal in both transcripts.

Recent technology on fluorescent proteins allows us to utilize a variety of colors (Shaner et al., 2005) in addition to the conventional colors, such as cyan (CFP), yellow (YFP), green (GFP), and red (DsRed). In particular, RFPs have greatly improved in the brightness and a variety of wavelengths for real-time and multi-color imaging (Shaner et al., 2004). For multi-color imaging without the fluorescent wavelength overlap, green and red are a more favorable combination than the conventional ones: CFP and YFP. To determine a useful RFP for optimal dual-color monitoring with GFP, we transfected plasmids encoding different RFPs. The brightness of the fluorescent proteins was tdTomato > mCherry ~ mOrange > DsRed; however, overexpression caused mOrange, tdTomato, and DsRed to slight overlap with the GFP image in our filter settings for the microscope (Supplementary Fig. 1). Therefore, mCherry was selected as the RFP in the following experiment.

To monitor the expression of miR-133 by using our retroviral system, the complementary sequence against miR-133 was inserted into the pMXCRGb plasmid between the GFP and CMV promoter to yield pMXCRGb[133] (Fig. 2B and C). Since miRNAs suppress the expression of target mRNA with complementarity, the expression of miR-133 can reduce the level of GFP whose mRNA possesses the

yellow). miR-133 activity was then quantified from the means of GFP intensity with RFP normalization as 30.6% and 13.8% after transfection of 10 and 100 nM of miR-133, respectively (Fig. 3B). When we transfected synthetic miR-1 as a negative control, the reduction of GFP was not observed (data not shown).

Next, we monitored endogenous miR-133 during myogenesis. Following 6 days of culturing in DM, the transduced cells successfully differentiated. The induction of differentiation suppressed the expression of GFP in C2C12 myoblasts that had been infected with MXCRGb[133] (Fig. 4A, middle). In particular, GFP fluorescence in myotubes was dramatically reduced (Fig. 4A, middle). RFP fluo-

rescence was unaffected by the same treatment; therefore, the suppression was specific to GFP. In contrast, the activity of GFP due to MXCRGb was unchanged by the induction of differentiation even in myotubes (Fig. 3A, bottom). GFP fluorescence was reduced only in C2C12 myoblasts containing MXCRGb[133], suggesting that the reduction was due to the expression of miR-133 in differentiating myotubes. Since myogenically differentiated cells formed largely fused cell aggregates, we could not apply these cells for quantitative analysis using flow cytometry. Therefore, we quantified fluorescence of differentiated fused cells by densitometry analysis of microscopic images (Supplementary Figure S3). This

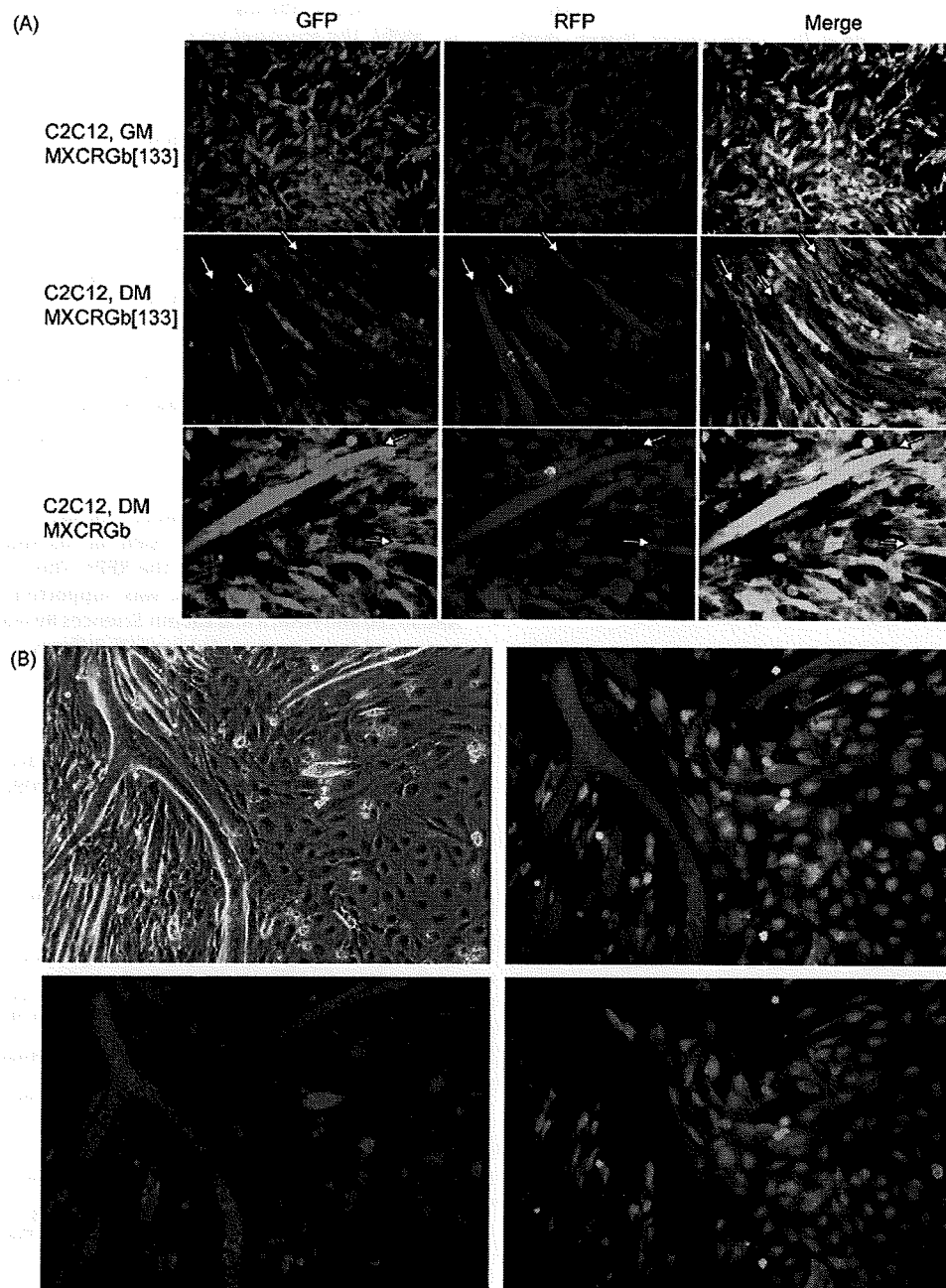


Fig. 4. Dual-color imaging of miR-133 expression in living myoblasts. C2C12 myoblasts were transduced with either the MXCRGb[133] or MXCRGb retroviral vector. (A) Strong expression of both GFP and RFP in C2C12 cells cultured in GM indicated that the retroviral vector was efficiently transduced (top). Six days after changing from GM to DM, GFP fluorescence in the differentiated MXCRGb[133] cells was reduced, while RFP fluorescence was retained (center). Fluorescence was unchanged in the case of both probes in MXCRGb cells. (B) Highly fused myotubes were observed 10 days after induction. Myotubes are indicated by arrows. Representative cell image is shown.

analysis showed striking changes in GFP compared with RFP signal upon myogenesis. Interestingly, GFP fluorescence in several cells was reduced prior to the myotube formation, indicating that miR-133 expresses and reduces the expression of target genes prior to myotube formation. Our analyses, in agreement with other reports, exhibited the dynamic change of miR-133 expression in living cells during myogenesis.

4. Discussion

4.1. Roles of miR-133 during myogenesis

Multiple factors participate in the step of myogenic differentiation with the regulation of muscle genes. In addition to the transcriptional regulation by transcription factors, post-transcriptional regulation by miRNA plays an important role in myogenic differentiation (Brennecke et al., 2005; Chen et al., 2006; Liu et al., 2008; Rao et al., 2006). Pairs of miR-1 and miR-133 are transcribed as bicistronic transcripts under the control of E-box, a family of MyoD transcription factors, and MEF2 transcription factor, an essential regulator of muscle development. MyoD and myogenin also modulate the transcription of miR-133 in skeletal muscle.

On the other hand, candidate target genes regulated by miR-133 are reported from a bioinformatics approach. One target is the serum response factor (SRF), which is a transcription factor that directly regulates the expression of miR-133. Transcription factors and miRNAs are suggested to regulate reciprocally in several physiological events (Chen et al., 2006; Hobert, 2008; O'Donnell et al., 2005). Muscle development involving miRNAs is presumed to be integrated in a complicated network since a single miRNA targets multiple mRNAs, and several co-expressed miRNAs may target a single mRNA with a synergetic effect (He et al., 2005; Stark et al., 2005). Our present results related to the role of miR-133 in myogenic differentiation are consistent with previous observations. We further clarified that the expression of miR-133 is induced prior to the formation of myotubes. Further analysis of miRNAs at the single cell level during differentiation will be necessary.

4.2. Retroviral vectors as a tool for sustainable and robust monitoring of miRNAs

In this study, we designed a retroviral vector system that enables real-time monitoring of miR-133 by using fluorescent proteins of different colors. The gene for GFP was connected with the target sequence of miR-133 at the 3' UTR, and the gene for RFP was independently expressed with a promoter other than GFP to ensure the transcriptional activity of the integrated site. Although internal ribosomal entry sites (IRESs) are known to express two or more proteins from a single transcript, IRES-connected transcripts have been reported to be affected by small dsRNA with perfect complementarity (Petersen et al., 2006). Therefore, we did not use IRES here. We observed that miR-133 regulates the expression of GFP; this regulation is not due to differences in the promoters used because the expression of GFP lacking miR-133 target sites was unaffected by the differentiation conditions. In addition to miR-133, we successfully detected other miRNAs, such as endogenous let-7 or adenoviral miRNAs (Kato, unpublished). Moreover, although repeated sequence of miRNA target may be concerned to trap and inactivate the endogenous miRNA, we found that C2C12 transduced with our retroviral vector successfully induced the myogenesis. It has been reported that over-expression of miRNA targets with perfect complementarity do not saturate miRNA regulation due to its catalytic activity of miRNA machinery (Brown et al., 2007).

Since the myogenic differentiation from myoblasts to myotubes takes a week, the reporter genes are required to be expressed sus-

tainably, aside from the plasmid transfection whose expression decreases after 3–4 days. The retroviral vector is integrated into the cellular genome and transgenes from the provirus are passed on to the daughter cells through doubling. However, since integration of the retroviral vector occurs randomly, it is possible that the expression of the transgenes of interest is unexpectedly affected by the control region of the cellular genome near the integration sites. Naldini's group reported that bidirectional expression of two distinct transgenes show excellent correlation regardless of the integration sites, although their vector was larger than ours due to the additional promoter and poly(A) signal. Moreover, the expression of miRNAs was indirectly monitored using a lentiviral vector based on HIV-1 by reducing GFP and a Δ LNGF receptor followed by fixation and fluorescent labeling with an anti- Δ LNGFR antibody (Brown et al., 2006). The lentiviral vector is amphotropic, namely, infectious to humans.

Our retroviral vector lacks such infectivity in humans due to its ecotropic packaging system. However, under biological safety restrictions, the retroviral system is a system of choice. When our retroviral vector is packaged with the vesicular stomatitis virus G (VSV-G) envelope, the virus can infect human cells, excluding non-dividing cells. Our retroviral vector also encodes two fluorescent proteins in tandem for monitoring miRNA expression by using living cells independent of integration sites of the provirus. The broader infectivity to vertebrates is promising for an extensive study of miRNAs in the *in vivo* tissues as well as *in vitro* cultured cells. Therefore, this retroviral system may be a useful tool for real-time monitoring of not only miR-133 in myogenic differentiation but also other miRNAs in various biological events.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2009.04.018.

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