Monitoring the Cellular Effects of HMG-CoA Reductase Inhibitors In Vitro and Ex Vivo

Iwona Cicha, Nicole Schneiderhan-Marra, Atilla Yilmaz, Christoph D. Garlichs, Margarete Goppelt-Strube

**Objective**—Inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase by statins and the subsequent reduction in Rho protein isoprenylation inactivates these important signaling molecules. The purpose of this study was to directly monitor statin effects on Rho proteins.

**Methods and Results**—We used biphasic Triton X-114 system, 1-dimensional isoelectric focusing, and 2D-electrophoresis for the separation of modified and nonmodified Rho proteins. These methods were evaluated in human fibroblasts treated with simvastatin. 2D-electrophoresis, which proved to be the most sensitive method, revealed 2 major spots of identical molecular weight but different isoelectric points, with the more basic spot representing the carboxymethylated form of RhoA. In control cells, 90% of RhoA was fully modified (carboxymethylated). After treatment with simvastatin, a significant shift toward the unmethylated form was observed, representing inhibition of isoprenylation, which is a prerequisite to further modification. Similar shifts were observed for Rac1 and Cdc42. In freshly isolated peripheral blood mononuclear cells, a shift toward nonmodified RhoA was observed after treatment with atorvastatin in vitro and in vivo. There was a significant increase in unmethylated RhoA in statin-treated individuals versus control individuals.

**Conclusion**—2D-electrophoresis is a sensitive method for detecting changes in the amount of nonisoprenylated Rho proteins, allowing monitoring the direct cellular effects of statins. *(Arterioscler Thromb Vasc Biol. 2004;24:1-6.)*

**Key Words:** RhoA ■ isoprenylation ■ carboxymethylation ■ HMG-CoA reductase ■ statins

Therapy with 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) is associated with lipid-lowering and plaque-stabilizing effects, resulting from their interference with cholesterol biosynthesis. However, recent experimental and clinical evidence indicate that the beneficial effects of statins extend beyond cholesterol reduction, involving improved plaque composition, decreased inflammation, and ameliorated endothelial function. These pleiotropic effects are mediated by the ability of statins to interfere directly with the post-translational modification of signaling proteins, particularly small GTP-binding proteins of the Ras and Rho families. Because the membrane localization and function of Ras and Rho depend on the attachment of isoprenoid intermediates to these proteins, by inhibiting protein isoprenylation, statin treatment leads to accumulation of the inactive forms of Ras and Rho in the cytoplasm. Although farnesylation of Ras proteins and geranylgeranylation of Rho proteins are equally affected by statins, many of the beneficial cardiovascular effects of statins are thought to be a result of their interference with Rho protein signaling.

As interference with the geranylgeranyl modification of Rho proteins is one of the earliest steps related to the direct cellular actions of statins, it appears desirable to determine the relative amount of modified versus nonmodified protein. Some of the isoprenylated proteins, such as Ras proteins, can be separated from their nonmodified form by high-resolution SDS-polyacrylamide gel electrophoresis (SDS-PAGE). However, for Rho family proteins, because of their specific type of post-translational modification, the separation of nonmodified and modified proteins cannot be assessed by SDS-PAGE.

Members of the Rho family, eg, RhoA, Rac1, and Cdc42, belong to a group of proteins that are characterized by the carboxyterminal amino acid pattern CaaX, where “a” represents an aliphatic amino acid and “X” may be any amino acid. These proteins are modified by 3 sequential modification steps. The first step of post-translational modification of Rho proteins is the transfer of a geranylgeranyl moiety to the cysteine residue by the respective transferase. Subsequently, the 3 terminal amino acids are cleaved by a prenylprotein-specific endoprotease. Finally, the terminal prenylcysteine is methylated by the isoprenylcysteine carboxyl methyltransferase (Figure 1). These modifications are not accompanied by a significant change in molecular mass. Because the
addition of the geranylgeranyl moiety is being compensated by the cleavage of the 3 terminal amino acids (mostly leucine, isoleucine, or valine), it is not possible to separate the nonmodified and the post-translationally modified forms by size using SDS-PAGE. Furthermore, because of their nonpolar nature, the removal of the terminal amino acids does not change the isoelectric point of the Rho proteins. However, carboxymethylation changes the isoelectric point of the protein as has been shown for Cdc42 and RhoA, with the methylated form being less acidic than the carboxylated form.

In the present study we investigated whether the difference in isoelectric point was a sensitive method to discriminate between the modified and nonmodified forms of the Rho family proteins before and after treatment with statins.

Methods

Methods are presented online at http://atvb.ahajournals.org.

Results

The human renal cell line TK173 was used as a model system to analyze the post-translational modification of Rho proteins. Western blot analysis of cellular proteins separated by 2-dimensional (2D) electrophoresis revealed 2 spots with an almost identical molecular weight of ~25 kDa, but different isoelectric points (Figure 2A). According to the literature, these spots represent the methylated and the unmethylated forms of RhoA.9 In control cells, >90% of RhoA was methylated. The acidic spot contained 2 overlapping forms of RhoA, the nonmodified form and the geranylgeranylated unmethylated form, which do not differ significantly in their size and isoelectric point. An increase in the nonmodified form was observed when the cells were incubated with either simvastatin (10 μmol/L, 18 hours) or an inhibitor of geranylgeranyltransferase (L841, 50 nmol/L, 18 hours) to prevent post-translational modification.

As observed previously, incubation of the cells with simvastatin or L841 increased the total synthesis of RhoA.10 Reprobing of the blot with an antibody directed against Cdc42 showed a similar distribution of the post-translationally modified forms of Cdc42 (Figure 2A). RhoA was still visible because both primary antibodies were of the same species. The isoelectric points of Cdc42 were shifted to a more alkaline pH than those of the RhoA forms, consistent
with data reported earlier by Backlund. Similar results were obtained with respect to Rac1, which overlapped with Cdc42 (data not shown).

In addition to the major acidic spot, some minor forms of RhoA shifted to more acidic pH were detected (arrows in Figure 2A, left). RhoA may be phosphorylated by kinases such as protein kinase A, suggesting that the minor acidic spots may represent phosphorylated forms of RhoA. To test this hypothesis, statin-treated fibroblasts were homogenized and incubated in vitro with alkaline phosphatase. As shown in Figure 2B, the most acidic spot disappeared, whereas the other smaller spot proved to represent a nonphosphorylated form of RhoA. The addition of mevalonate or geranylgeranylpyrophosphate to simvastatin-treated fibroblasts almost completely prevented the generation of the acidic unmethylated forms of RhoA (Figure 2C).

The long-term effect of statins was evaluated by culturing fibroblasts for 7 days in the presence of different concentrations of simvastatin. As the metabolic fate of simvastatin in cell culture is not known, the cells were treated with simvastatin every day between the medium changes, nominally increasing the concentration of simvastatin 3-fold. In the presence of 1 μmol/L simvastatin, the acidic forms of RhoA were predominant, and the shift was still detectable at 10 nmol/L simvastatin, a concentration that is close to the levels expected in vivo (Figure 3).

To take advantage of the difference in isoelectric points, samples were also directly loaded on 1-dimensional IEF gels (Figure I, available online at http://atvb.ahajournals.org). The shift from the methylated to the nonmethylated form was clearly visible in samples treated with simvastatin and L841, but not in control samples or samples treated with both simvastatin and mevalonate. However, compared with the 2D analysis, this method proved to be less sensitive with no detectable unmethylated form in control samples. Therefore, all further analyses were performed using 2D electrophoresis.

Another method to separate geranylgeranylated RhoA from nonmodified RhoA partitions the nonmodified RhoA into the aqueous phase of the biphasic Triton X-114 system. After treatment of the cells with simvastatin, nongeranylgeranylated RhoA became detectable in the aqueous phase when the protein was analyzed by standard SDS-PAGE (Figure IIa, available online at http://atvb.ahajournals.org). The aqueous and detergent phases had to be analyzed on 2 different 2D gels that were run in parallel. Western blotting was also performed using the same antibody and detection of the ECL signal with one film to allow semi-quantitative analysis. The detergent-containing phase of control cells contained approximately equal amounts of methylated and unmethylated forms of RhoA, indicative of demethylation during the incubation of the homogenates (Figure IIb). Only ~10% of total RhoA was detectable in the aqueous phase, which was acidic and thus unmethylated. On treatment with simvastatin, the nonmodified form of RhoA was extracted into the aqueous phase. However, a considerable amount of nonmodified RhoA remained in the detergent phase, thus demonstrating one of the limitations of the separation by the Triton X-114 system.

To relate the results of the in vitro experiments to the therapeutic situation, PBMCs obtained from healthy volunteers were investigated. To avoid demethylation caused by inappropriate handling of the samples, PBMCs were extracted immediately after blood collection, and the protein pellets were frozen at −80°C until analysis. In these mononuclear cells, ~80% of RhoA was methylated (Figure 4A; Co in vivo). Culturing of PBMCs for 24 hours shifted the ratio from the methylated to the unmethylated form (Figure 4A; Co in vivo compared with Co in vitro). As shown in Figure 4A, incubation with 1 or 10 μmol/L atorvastatin for 24 hours interfered with geranylgeranylation of de novo synthesized protein, leading to the increase in the proportion of unmethylated RhoA in vivo compared with Co in vitro.

Monoclonal antibodies were investigated. To avoid demethylation caused by inappropriate handling of the samples, PBMCs were extracted immediately after blood collection, and the protein pellets were frozen at −80°C until analysis. In these mononuclear cells, ~80% of RhoA was methylated (Figure 4A; Co in vivo). Culturing of PBMCs for 24 hours shifted the ratio from the methylated to the unmethylated form (Figure 4A; Co in vivo compared with Co in vitro). As shown in Figure 4A, incubation with 1 or 10 μmol/L atorvastatin for 24 hours interfered with geranylgeranylation of de novo synthesized protein, leading to the increase in the proportion of unmethylated RhoA in vivo compared with Co in vitro. In vivo, the shift from fully modified, ie, methylated RhoA to nonmodified RhoA, was clearly visible and the more acidic forms became detectable (Figure 4A). Furthermore, PBMCs of 3 different healthy volunteers were analyzed and compared with samples obtained from subjects who were prophylactically treated with simvastatin (10 mg/d) or atorvastatin (20 mg/d) for asymptomatic familial hypercholesterolemia. The percentage of nonmodified RhoA in healthy subjects was 24.1 ± 6.3 (means ± SD) and increased to 42% and 49%, respectively, in statin-treated subjects.

Based on these studies, which showed the applicability of the method to peripheral blood mononuclear cells, 10 healthy volunteers were enrolled in a subsequent study. The subjects took 40 mg/d atorvastatin for 5 days. Volunteers served as their own controls. Treatment with atorvastatin reduced the cholesterol levels in all but one volunteer by >20% (Figure 4B). However, analysis of the ratio of methylated and
A

![Image](https://example.com/image1.png)

**Discussion**

Statins have been shown to exert many beneficial effects on vascular functions. For example, statins increase endothelial nitric oxide synthase expression, decrease smooth muscle cell proliferation, reduce matrix metalloproteinase activity and tissue factor production by macrophages, increase fibrinolytic activity, and have immunomodulatory and anti-inflammatory actions such as increasing resistance to complement and decreasing dendritic cell maturation. Many of these effects are reversed only with geranylgeranyl pyrophosphate and not with farnesyl pyrophosphate, suggesting that inhibition of Rho isoprenylation by statins is the predominant mechanism by which statins exert their anti-atherogenic activities. The members of the Rho family collectively regulate the actin cytoskeleton, serving specific functions in maintaining cell shape, motility, secretion, and proliferation. The inhibition of Rho proteins is one of the earliest steps in the direct cellular activity of statins; therefore, it is important to monitor the changes in the amounts of modified and non-modified forms of protein.

In the present study, we demonstrate that separation by 2D electrophoresis is a useful method to determine the interference of statins with the geranylgeranyl modification of Rho proteins not only in vitro but also after treatment ex vivo. The advantage of this method is its independence of any specific target of statins. It detects changes in isoprenylation, which account for the majority of the direct cholesterol-independent actions of these drugs.

The prerequisite for the analysis was the observation that, depending on the cell type, 80% to 90% of geranylgeranyl-modified Rho proteins are also carboxymethylated. In contrast to the irreversible cleavage of the C-terminal amino acids, methylation is principally reversible. In our experiments, demethylation was observed on incubation of cellular homogenates, which was not unexpected because of the presence of esterases, which hydrolyze the methyl esters. Accumulation of the unmethylated form was also observed when PBMCs were cultured in vitro for 24 hours. Given the long incubation time, the shift may be caused by interference with carboxymethylation which, among other mechanisms, is regulated by the level of S-adenosyl-methionine within the cells.

Treatment of cells in vitro with high concentrations of statins resulted in an increase in the acidic forms of the Rho protein, which reached >90% of the total RhoA amount. The shift could not be attributed to interference with the methylating step, because it was prevented by coincubation of the cells with mevalonate, the precursor of geranylgeranyl pyrophosphate. This finding suggests the shift is secondary to the direct inhibition of HMG-CoA reductase by statins. Furthermore, a similar shift was also observed when the geranylgeranyl transferase was directly inhibited. Analysis of the phases of the Triton X-114 separation method confirmed that the acidic spots represented the form of RhoA that was not geranylgeranyl-modified. In addition, we demonstrated the limitations of the Triton method because a considerable amount of nonmodified protein was found in the detergent phase, which was supposed to contain only modified RhoA. Taken together, the in vitro data confirmed that the statin-mediated increase in the acidic forms of RhoA was attributable to Rho proteins that were not modified by isoprenylation. The isoelectric points of the 3 members of the Rho family, RhoA, Rac1, and Cdc42, differ slightly, but the shift observed for all 3 proteins was comparable. This finding was not unexpected given that the 3 proteins have similar terminal
amino acids, consisting of 2 leucines and valine, isoleucine, or leucine.

Based on the differences in isoelectric points, we tried to separate the modified forms of the Rho proteins by 1-dimensional isoelectric focusing using commercially available gels. However, these gels proved to be less effective than the 2D electrophoresis. Because of the larger area of the individual bands compared with the spots in the 2D analysis, the sensitivity was decreased. The minor acidic spots were not detectable by this type of analysis. Furthermore, separation proved to be less reproducible compared with the 2D analysis. Therefore, to get reliable results, each sample was separated by 2D electrophoresis.

Rho proteins are major regulators of the actin cytoskeleton, and cytoskeletal changes can affect intracellular transport, mRNA stability, and gene transcription. Thus, given the importance of Rho proteins in the regulation of cell growth, gene expression, and organization of the cytoskeleton, a complete inhibition of Rho modification should not be expected after treating patients with statins. Consistent with this, we found a change in the ratio of isoprenylated versus nonmodified form when mononuclear cells from statin-treated individuals were compared with the cells of healthy volunteers. However, even in the statin-treated individuals, >40% of Rho proteins remained fully modified, ie, in the carboxymethylated form. The shift toward unmethylated form was observed in mononuclear cells in vivo after 1 week of treatment with a moderate dose of atorvastatin (40 mg/d) and after prolonged treatment with low doses of simvastatin (10 mg/d) or atorvastatin (20 mg/d).

In a controlled study with healthy volunteers taking 40 mg/d atorvastatin over 5 days, the size of the more acidic spot proved to be the most sensitive measure of the effect of atorvastatin. It was more than doubled in 6 of 10 volunteers, whereas the other subjects it did not show any significant change. This interindividual variability may be caused by the fact that atorvastatin is supposed to inhibit HMG-CoA reductase in the liver, so the systemic levels of this lipophilic compound vary considerably between individuals.12 Extending our data to the clinical situation, the differential response of the volunteers suggests that individual patients will benefit to different degrees from the pleiotropic effects of statins, independently of the lipid-lowering effect. The increase in nonisoprenylated inactive form of RhoA in PBMCs has been linked to the atheroprotective effects of statins on these cells, such as downregulation of plasminogen activator inhibitor-1 synthesis, decreased transcription of matrix metalloproteinases and adhesion molecules in monocytes, decreased monocyte–endothelial cell interactions, and inhibition of activation and proliferation of T lymphocytes. The most responsive patients will be expected to benefit from lipid-lowering and from pleiotropic atheroprotective effect of statins. However, in patients showing little inhibition of Rho isoprenylation in circulating cells of immune system, additional therapy may be needed to attenuate the inflammatory responses in atherosclerosis. Thus, monitoring of RhoA isoprenylation may be a useful tool for the prediction of the possible pleiotropic benefits in statin-treated patients.

In conclusion, the separation by 2D electrophoresis is a sensitive method to monitor the inhibition of RhoA isoprenylation, which is responsible for the majority of pleiotropic effects of statins. Studies with larger numbers of patients will be needed to determine additional variables, which might affect the post-translational modification of Rho proteins.

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Figure I. Isoelectric focusing of Rho proteins after treatment with statins *in vitro*.

Human renal fibroblasts were incubated with simvastatin (10 µmol/L), the geranylgeranyl transferase inhibitor L841 (50 nmol/L) or mevalonate (500 µmol/L) as indicated, for 18 h. Cellular protein was separated by one-dimensional IEF. RhoA was detected by Western blotting.
A. Human renal fibroblasts were incubated with simvastatin (10 µmol/L). Equal amounts of cellular protein were separated by the Triton X-114 extraction method. Aliquots of the aqueous and the detergent phase were analyzed by SDS PAGE. RhoA was detected by Western blot analysis. Two separate culture dishes were analyzed in parallel for each condition.

B. For 2D analysis, the aqueous and detergent phases were ethanol-precipitated. Precipitates were dissolved in rehydration buffer and loaded on the IEF stripes. RhoA was detected by Western blot analysis after 2D separation of the proteins.
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Materials and Methods

Reagents
RPMI 1640 medium, DMEM, penicillin/streptomycin were from PAA Laboratories, Pasching, Austria. Phosphate-buffered saline (PBS) and fetal calf serum (FCS) were purchased from Biochrom, Berlin, Germany. Mevalonic acid lactone was converted to sodium mevalonate as described by Essig et al [1]. The specific inhibitor of geranylgeranyltransferase, L841491 (L841), and simvastatin were kindly provided by MSD, Sharp and Dohme, Munich, Germany [2]. Simvastatin was dissolved in ethanol and activated as described by Jakobisiak et al. [3]. Atorvastatin was kindly provided by Pfizer, Karlsruhe, Germany.

A mouse monoclonal antibody directed against human RhoA was purchased from Santa Cruz, Heidelberg, Germany. Mouse anti-human Cdc42 and Rac1 antibodies were from BD Transduction Laboratories, Heidelberg, Germany. Secondary horseradish peroxidase-conjugated anti-mouse IgG was obtained from Amersham, Freiburg, Germany.

Fibroblast Cell Culture
Immortalized human renal fibroblasts were kindly provided by G. Muller, Goettingen [4], Germany. The cells were characterized in detail and shown to maintain the characteristics of primary fibroblasts [4]. Culturing of the cells was done as previously described [5,6].

Phosphatase Treatment
After stimulation with simvastatin, fibroblasts were collected in 50 mM Tris/HCl, pH 7.9, 10 mmol/L MgCl₂, 100 mmol/L NaCl and 1 mmol/L dithiotreitol. Cellular homogenates were obtained by three freeze-thaw cycles in liquid nitrogen followed by three sonification cycles, 15 sec each. Cellular proteins (160 µg) were incubated with 30 U alkaline phosphatase (calf intestinal phosphatase, Sigma, Munich, Germany), for 1 h at 37 °C. The proteins were ethanol-precipitated before analysis by isoelectric focusing.

Separation of Isoprenylated and Non-Isoprenylated RhoA
Isoprenylated RhoA was determined essentially as described by Chappell et al. [7]. After treatment with or without simvastatin, whole cell lysates were prepared in lysis buffer (1 mmol/L Na₃PO₄, 50 mmol/L Heps, 5 mmol/L MgCl₂, 150 mmol/L NaCl, 0.05% SDS, 1%
Triton X-100). Cells were sonicated 2 x 10 s at 50% output (Branson sonifier), and debris was removed by centrifugation. Lysate containing about 450 µg protein was mixed with an equal volume of 4% Triton X-114 and warmed to 37 °C for 3 min. Separation of a Triton-rich lower phase containing the isoprenylated form of RhoA and an upper buffer phase containing the non-modified form was carried out at room temperature. Before analysis by SDS-PAGE, the protein of the lower phase was precipitated by ethanol, and the pellet was resuspended in 20 mmol/L Tris/HCl pH 8.0 and 1 mmol/L EDTA. For isoelectric focusing (IEF) the total upper and lower phase were precipitated.

**Isoelectric Focusing**

One-dimensional IEF was performed according to the manufacturer’s instructions (Invitrogen, Karlsruhe, Germany). Briefly, cell pellets were resuspended in Novex™ 3-10 IEF sample buffer (Invitrogen) and sonicated twice for 10 s. After measuring the total protein concentration with Bradford reagent (BioRad, Munich, Germany), 20 µg of protein was loaded per lane on one-dimensional Novex™ IEF gel pH 3-10. Electrophoresis was run using the buffer system specified by the manufacturer.

**Two-Dimensional Gel Electrophoresis (2D Electrophoresis)**

Cell pellets were resuspended in lysis buffer containing 9 mol/L urea, 4% CHAPS, 1% dithiothreitol (DTT), and 2% Biolyte 3-10 (Amersham) followed by sonication. Protein concentration was determined in each sample using BioRad protein assay. Immobiline DryStrip gels pH 3-10 NL (Amersham) were rehydrated for 12 h at 20 °C using rehydration solution containing 8 mol/L urea, 1% CHAPS and 0.4% DTT. One hundred µg of total protein was loaded with rehydration solution. First-dimension isoelectric focusing of proteins was run at 20 °C on a IGPhor isoelectric focusing unit with the voltage program specified by the manufacturer. Following the first-dimension separation, proteins were separated on 12% SDS-PAGE gels.

**Western Blot Analysis**

For Western blot analysis, the proteins were transferred to polyvinylidene difluoride membranes. After blocking with 5% horse serum in Tris-buffered saline containing 0.1% Tween 20, RhoA was detected using anti-RhoA antibody (1:200) overnight at 4 °C, followed by the secondary anti-mouse IgG (1:10 000) for 1 h. Anti-Cdc42 and anti-Rac1 antibodies were used at dilutions of 1:200 and 1:1500, respectively. Immunoreactive proteins were visualized using enhanced chemiluminescence system (ECL-Plus, Amersham). For quantification purposes, densitometric measurement was performed using the image analysis software from Bioprofil (Froebel, Germany). To analyze the minor spots,
luminescence measurements were performed using a luminescent image analyzer (LAS-1000, Fujifilm) and Aida 2D image analysis software (Raytest, Berlin, Germany).

**Preparation of Peripheral Blood Mononuclear Cells (PBMCs)**

Venous blood from adult volunteers was drawn on acid-citrate-dextrose buffer. Three volunteers took no medication, and two subjects were taking low prophylactic doses of statins (simvastatin 10 mg/d or atorvastatin 20 mg/d). One healthy subject was asked to take 40 mg of atorvastatin per day for 1 week. Blood from this volunteer was drawn before the start of medication, on day 2 and on day 7 after the start of medication. PBMCs were isolated immediately after collecting blood.

In a subsequent clinical study, 10 healthy volunteers (mean age 31 ± 7.1 y) were given 40 mg/d atorvastatin for 5 consecutive days. Blood was drawn before the start of the study and after 5 days of treatment. PBMCs were isolated within 1 h of blood collection. Total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglyceride levels in the sera were measured before and after the study. The protocol was approved by the local ethics committee, and written informed consent was obtained from all the volunteers.

PBMCs were separated by density gradient centrifugation using Vacutainer cell preparation tubes (CPT, Becton Dickinson). After washing the cells 3 times in PBS, the pellets were frozen at -80°C until the analysis. For *in vitro* experiments, PBMCs were washed twice in PBS and once in RPMI 1640 medium, and resuspended in RPMI 1640 supplemented with 2 mmol/L glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% FCS. The cells were seeded on 60 mm diameter culture dishes at the density of 6 x 10^6 cells per dish. Viability of cells was greater than 95% as estimated by Trypan blue exclusion. The cells were cultured for 24 h with presence or absence of 10 µmol/L atorvastatin and 100 µmol/L mevalonate at 37 °C in a humidified 5% CO_2 incubator. After harvesting, the cells were washed with PBS, and the pellets were frozen at -80°C until analysis.

**References**


