A Novel Assay System for Macrophage-activating Factor Activity Using a Human U937 Cell Line

MAMI ISHIKAWA[1], TAKAHIRO INOUE[1], TOSHIO INUI[3,4,5,6], DAISUKE KUCHIIKE[3,4], KENTARO KUBO[4], YOSHIHIRO UTO[3] and TAKAHITO NISHIKATA[1,2]

[1]*Frontiers of Innovative Research in Science and Technology (FIRST) and*
[2]*Frontier Institute for Biomolecular Engineering Research (FIBER), Konan University, Kobe, Japan;*
[3]*Department of Life System, Institute of Technology and Science, Graduate School, University of Tokushima, Tokushima, Japan;*
[4]*Saisei Mirai Cell Processing Center, Osaka, Japan;*
[5]*Kobe Saisei Mirai Clinic, Kobe, Japan;*
[6]*Inui Immunotherapy Clinic, Osaka, Japan*

Abstract. *Background: Macrophages play important roles in antitumor immunity, and immunotherapy with the group-specific component protein-derived macrophage-activating factor (GcMAF) has been reported to be effective in patients with various types of cancers. However, in macrophage research, it is important to properly evaluate macrophage activity. Materials and Methods: U937 macrophages were induced by 12-O-tetradecanoyl-13-phorbolacetate (TPA). The phagocytic activity of macrophages was evaluated as the internalized beads ratio. The MAF activity was assessed at 30 min after MAF addition as the activation ratio. Results: We established a novel assay for phagocytic activities using differentiated U937 macrophages. Conclusion: The novel protocol was simple and rapid and was sensitive for GcMAF. This protocol should be useful not only for basic studies, such as those on molecular mechanisms underlying macrophage activation, but also for clinical studies, such as assessment of GcMAF activity prior to clinical use.*

Innate immunity is at the frontline of the host defense system against pathogens, such as bacteria and viruses. Phagocytic cells, such as macrophages and neutrophils, internalize and destroy pathogens and release cytokines. Moreover, macrophages are important in antitumor immunity (1).

The group-specific component (Gc protein), which is known as Gc globulin or vitamin D3-binding protein, is a serum protein and precursor for macrophage-activating factor (MAF) (2). In an inflammatory response, Gc protein is hydrolyzed by an inducible β-galactosidase of inflammation-primed B-lymphocytes and neuraminidase of T-lymphocytes to yield MAF bearing N-acetylgalactosamine (GalNAc) moiety as the remaining sugar (GcMAF) (3). It has been reported that immunotherapy with GcMAF is effective in patients with thymic carcinoma (4), prostate cancer (4), metastatic colorectal cancer (5), and non-anemic HIV-infected patients (6). In spite of extensive clinical studies, the mechanisms underlying the biological function of GcMAF, including how GcMAF activates macrophages, are poorly understood.

When conducting macrophage research, it is important to evaluate macrophage activity properly. Phagocytic activity and the amounts of nitric oxide and tumor necrosis factor-α are prevailing indices of macrophage activity (7). Although phagocytic activity is an ideal index, phagocytosis is a complex biological reaction and the outcomes of the assay can vary according to the assay protocols. Some prevailing protocols of the MAF assay use mouse peritoneal macrophages, a mouse macrophage-like cell line (RAW264.7), or a human monocytic leukemia cell line (THP-1) as effector cells and opsonized sheep-red blood cells, latex beads, or zymosans as target substances (8-13). The results obtained from the repertoire of such assay systems are difficult to compare, necessitating a standardized MAF assay for macrophage research. Furthermore, as general methods are time consuming, involving the fixation followed by histological staining, such as Giemsa stain, a standardized method needs to be rapid and simple.

This article is freely accessible online.

Correspondence to: Takahito Nishikata, Frontiers of Innovative Research in Science and Technology (FIRST), Konan University, 7-1-20, Minatojima-minamimachi, Chuo-ku, Kobe 605-0047, Japan. Tel: +81 783031349, Fax: +81 783031495, e-mail: nisikata@konan-u.ac.jp

Key Words: MAF, macrophage-activating factor, GcMAF, Gc protein-derived macrophage-activating factor, phagocytic activity, U937 cell line.

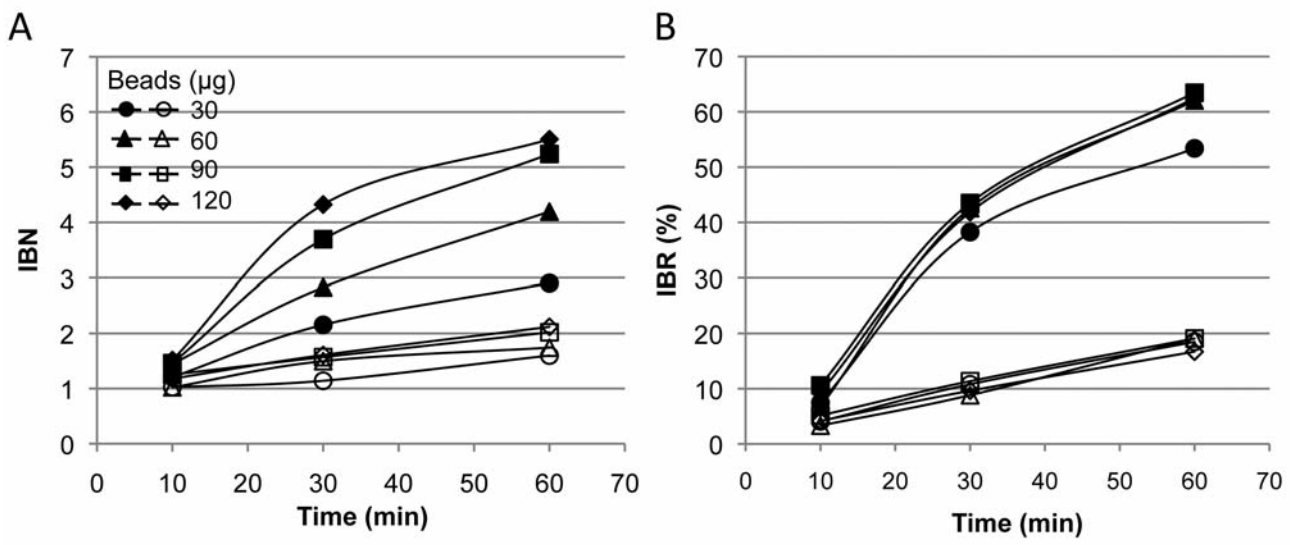

Figure 1. *Difference in phagocytic activity of U937 macrophages as a result of the amount of beads. Phagocytic activity was evaluated by the internalized beads number (IBN) (A) and the internalized beads ratio (IBR) (B). Details of these calculations are described in the Materials and Methods Section. Macrophages were cultured in the presence (closed symbols) or absence (open symbols) of Gc protein-derived macrophage-activating factor. Error bars represent SD (n=3).*

The human leukemia monocyte lymphoma cell line (U937) is easily grown in suspension culture and can be differentiated into macrophage-like cells by the treatment with a phorbol ester, such as 12-O-tetradecanoyl-13-acetate (TPA) (14). Thus, U937 is a good source for homogeneous macrophage-like cells. In the present study, we established a novel assay system for macrophage phagocytic activity using the human U937 cell line. We propose this novel method as a standardized method for assessing activity of MAF, particularly that of GcMAF.

Materials and Methods

Cells and cell culture. U937 cells were obtained from Summit Pharmaceutical (Tokyo, Japan). U937 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 3% L-glutamine, and 10% sodium hydrogen carbonate. Cells were cultured in a 5% CO_2/95% air fully-humidified atmosphere at 37˚C. To induce differentiation into macrophage-like cells, U937 cells were seeded onto 35-mm culture dishes (5.0×10^5 cells/dish) and incubated for 72 h with 10 ng/ml TPA (Sigma-Aldrich, St. Louis, MO, USA). In the case of high macrophage-like cell densities, 5×10^6 cells were induced for differentiation in 100-mm culture dishes. The differentiated macrophage-like cells were trypsinized, transferred into 35-mm culture dishes, and pre-cultured for three days with 10 ng/ml TPA.

Phagocytosis assay. The differentiated macrophage-like cells were pre-treated with serum-free RPMI-1640 medium for 120 min before GcMAF treatment. The medium was then changed to serum-containing normal RPMI-1640 medium containing 5 μg/ml GcMAF and 90 μg magnetic beads (Dynabeads® Protein G; Invitrogen, Oslo, Norway). Macrophages were photographed under a bright field microscope at 10, 30, and 60 min after GcMAF addition and counted for internalized and non-internalized beads. Phagocytic activities of macrophage were evaluated as the internalized beads ratio (IBR) and the internalized beads number (IBN). The activity of GcMAF was evaluated as the GcMAF activation ratio (AR) at 30 min after GcMAF addition. These indices were calculated by the following formulas:

IBR (%)=(number of internalized beads within the photograph)/(number of all beads within the photograph) ×100

IBN=(number of internalized beads within the photograph)/(number of macrophages within the photograph)

AR=(internalized beads ratio with GcMAF)/(internalized beads ratio without GcMAF)

Results

In this study, phagocytic activity was assessed by the internalization of beads. Phagocytic activity of macrophages and the MAF activity were evaluated as the IBR and AR, respectively, at 30 min after MAF addition. Details of these calculations are described in Materials and Methods. We propose the IBR as a robust index of phagocytic activity.

We examined the culture conditions of macrophages just before adding GcMAF (Table I). In general, differentiated U937 macrophages are cultured with RPMI-1640 medium supplemented with 10% serum for 72 h (14). Initially, we examined the phagocytic activity of macrophages under these conditions with and without GcMAF. IBR values with and without GcMAF were 39.9±2.8% and 27.8±5.4%,

respectively, and the AR was 1.5±0.2. When macrophages were incubated with serum-free medium for 72 h, IBR values with and without GcMAF were 27.5±6.3% and 6.8±1.7%, respectively. The AR was 4.1±0.2. Incubation with serum-free medium increased sensitivity to GcMAF. We added a 2-h incubation with serum-free medium after the 72 h serum-containing culture condition. Under these conditions, the IBR values with and without GcMAF were 39.5±9.0% and 7.1±1.9%, respectively. The AR was 5.5±0.8. The short incubation with serum-free medium effectively sensitized the differentiated U937 macrophages to GcMAF.

Using the sensitized macrophages, we evaluated the appropriateness of the IBR as an index of phagocytic activity and determined the ideal amount of beads for use in the assay protocol. Different amounts (30 to 120 μg) of beads were added to 5×10^5 cells of U937 macrophages plated in 35-mm dishes. As shown in Figure 1A, IBN increased according to the amount of beads, suggesting that increasing the amount of beads elevated the chance of engulfment by macrophages. On the other hand, within this range of beads, IBRs converged to similar values (Figure 1B). This might have been because the chance of engulfment was offset by the chance of escaping from engulfment. The amount of beads in the assay protocol was adjusted to 90 μg.

In order to determine the effect of the number of macrophages, we compared the ARs between 5.0×10^5 and 5.0×10^6 cells in 35-mm dishes. As shown in Table II, although the IBR was increased when using higher cell numbers, the AR decreased from 5.4±2.2 to 2.2±1.0. Thus, the preferable number of macrophages in a 35-mm dish was 5.0×10^5 cells.

The effect of GcMAF on differentiated U937 macrophages was verified by light microscopy (Figure 2). The spherical macrophage-like cells adhered to the bottom of the dish, and some extended lamellipodia. When U937 macrophages were cultured without GcMAF, only a few beads were internalized by each macrophage. In this situation, the beads that were not engulfed by macrophages were scattered on the bottom of the culture dish. On the other hand, when U937 macrophages were cultured with GcMAF, macrophages start engulfing beads only 10 min after addition of GcMAF. Sixty min after the addition of GcMAF, most of the beads had formed clusters within the macrophages. Although some clusters were formed within the flattened cell body and seemed to exist extracellularly, all of the clusters were formed within macrophages. Thus, it was easy to discriminate between internalized beads and beads that had escaped engulfment.

Finally, we analyzed the dose dependency of GcMAF in our novel MAF assay protocol. The MAF assay was carried-out in the presence of 0.005, 0.05, 0.5, 5, 50, or 500 μg/ml GcMAF. Over this concentration range, the IBR showed monotonic increase for at least 1 h (Figure 3A). The maximum AR was 5.8±0.5 at 5 μg/ml GcMAF (Figure 3B). These results showed the high sensitivity and relatively wide effective range of our MAF assay protocol. Thus, we propose this novel assay protocol for phagocytic activities as a suitable system for assessing MAF activity.

Table I. *Difference in the phagocytic activity of U937 macrophage due to culture conditions.*

Culture conditions	IBR (%)		AR
	Without GcMAF	With GcMAF	
Serum-containing medium	27.8±5.4	39.9±2.8	1.5±0.2
Serum-free medium	6.8±1.7	27.5±6.3	4.1±0.2
Serum-free medium (2 h)	7.1±1.9	39.5±9.0	5.5±0.8

IBR: Internalized beads ratio (%) AR: activation ratio. Data are described as the mean±SD (n=3).

Table II. *Difference in the phagocytic activity of U937 macrophage due to number of cells.*

Number of cells	IBR (%)		AR
	Without GcMAF	With GcMAF	
5×10^5	9.3±4.9	41.6±9.2	5.4±2.2
5×10^6	37.9±18.6	69.2±16.0	2.2±1.0

IBR: Internalized beads ratio (%) AR: activation ratio. Data are described as the mean±SD (n=3).

Discussion

In the present report, we established a novel assay protocol for assessing MAF activity using differentiated U937 macrophages. It is a simple and rapid MAF assay system and is sensitive for GcMAF.

In this protocol, there are three major innovative points. Firstly, we invented novel indices, the IBR for phagocytic activity and the AR for MAF activity. Robustness of the IBR was clearly demonstrated. Secondly, we used magnetic beads as target substances. In the general phagocytic assay, plastic beads, such as latex beads, are used (11, 12). Because of the low density of these plastic beads [~1.05 g/cm^3; (15)], they gradually settle on the bottom of the dish. Thus, the internalization of the beads is dependent on both phagocytic activity and the speed at which the beads settle. With magnetic beads (~2.0 g/cm^3), the beads almost completely settled within a minute, and the speed of internalization of the beads was a reflection of the phagocytic activity of the macrophages. Not only is this a great advantage for properly evaluating phagocytic activity but it also makes it possible to

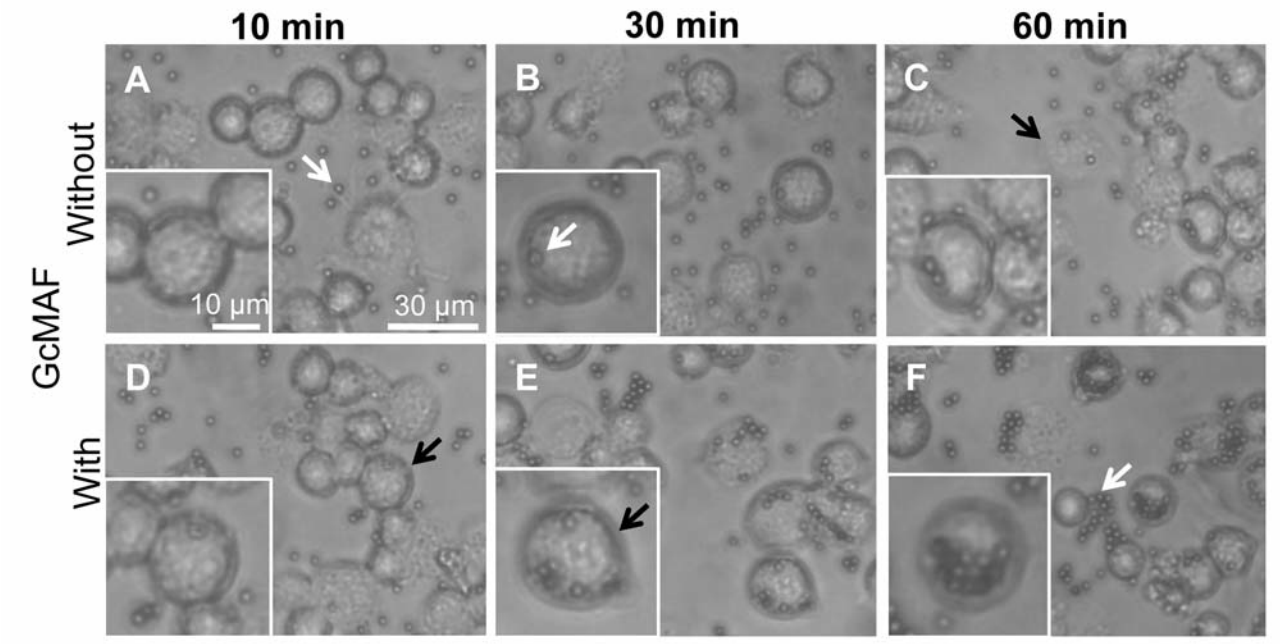

Figure 2. *Photomicrographs of macrophages engulfing beads. Macrophages incubated without (A-C) or with (D-F) Gc protein-derived macrophage-activating factor (GcMAF) at 10 min (A, D), 30 min (B, E), and 60 min (C, F) after the addition of beads were photographed under bright field microscopy. Insets show enlarged images. Black arrows indicate macrophages. White arrows indicate beads.*

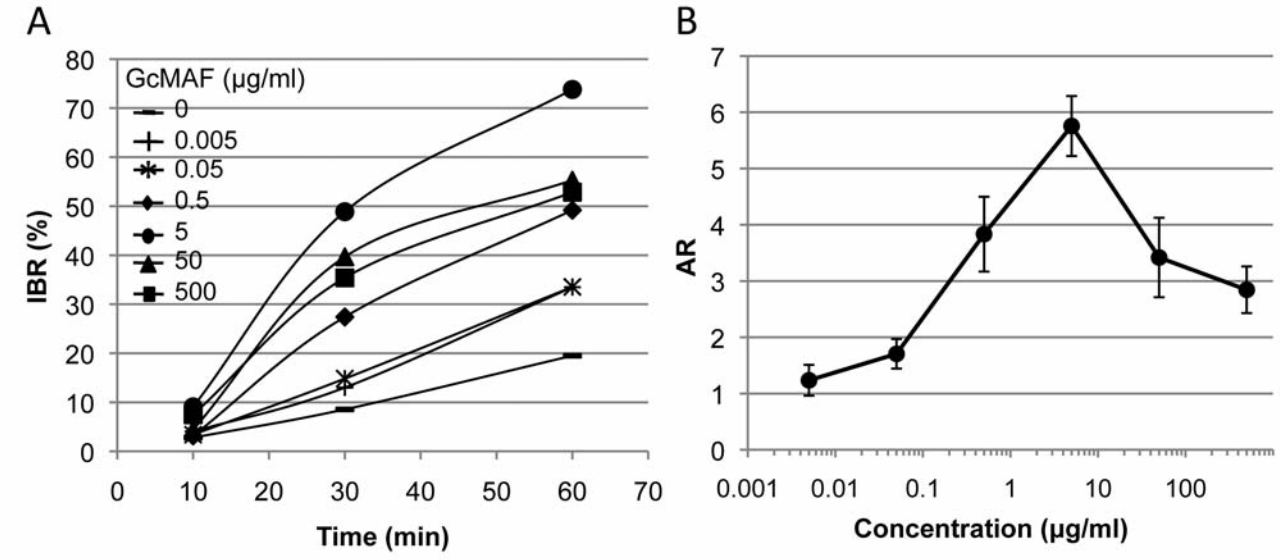

Figure 3. *Difference in phagocytic activity of U937 macrophages as a result of Gc protein-derived macrophage-activating factor (GcMAF) concentration. Macrophages were cultured in the presence of 0.005, 0.05, 0.5, 5, 50, or 500 μg/ml GcMAF. Phagocytic activity was evaluated by the internalized beads ratio (IBR) (A) and the GcMAF activation ratio (AR) (B). Error bars represent SD (n=3).*

reduce the total assay time. Thirdly, in this protocol, we photographed engulfing macrophages and counted the numbers of internalized beads. This method facilitates observation of the time course of the activating macrophages. Furthermore, it also facilitates the design of more advanced assay protocols using functional beads, such as beads labeled with a pH-sensitive fluorescent probe (16).

The key point of our novel MAF assay protocol is the use of human leukemia monocyte lymphoma cell line, U937. These cells can be easily grown in suspension culture and can

be differentiated into macrophage-like cells by treatment with a phorbol ester, such as TPA (14). This permits the preparation of stable macrophage-like cells of a consistent quality. This is one of the most important factors for standardization of the protocol. Moreover, we were able to sensitize the U937 macrophages by a very simple method, 120 min of treatment with serum-free medium. This treatment was very important for increasing the sensitivity of this protocol. Furthermore, this sensitization phenomenon provides some insight into the molecular mechanisms underlying macrophage activation by MAFs, including GcMAF.

References

1 Mantovani A, Bottazzi B, Colotta F, Sozzani S and Ruco L: The origin and function of tumor-associated macrophages. Immunol Today *13*: 265-270,1992.

2 Yamamoto N and Homma S: Vitamin D_3 binding protein (group-specific component) is precursor for the macrophage-activating signal factor from lysophosphotidylcholine-treated lymphocytes. Proc Natl Acad Sci USA *88*: 8539-8543, 1991.

3 Yamamoto N and Kumashiro R: Conversion of vitamin D_3-binding protein (group-specific component) to macrophage-activating factor by the stepwise action of beta-galactosidase of B-cells and sialidase of T-cells. J Immunol *151*: 2794-2802, 1993.

4 Inui T, Kuchiike D, Kubo K, Mette M, Uto Y, Hori H and Sakamoto N: Clinical experience of integrative cancer immunotherapy with GcMAF. Anticancer Res *33*: 2917-2920, 2013.

5 Yamamoto N, Suyama H, Nakazato H, Yamamoto N and Koga Y: Immunotherapy of metastatic colorectal cancer with vitamin D-binding protein-derived macrophage activating factor, GcMAF. Cancer Immunol Immunother *57*: 1007-1016, 2008.

6 Yamamoto N, Ushijima N and Koga Y: Immunotherapy of HIV-infected patients with Gc protein-derived macrophage activating factor (GcMAF). J Med Virol *81*: 16-21, 2009.

7 Mohamad SB, Nagasawa H, Sakai H, Uto Y, Nagasawa Y, Kawashima K and Hori H: Gc-Protein-derived macrophage activating factor (GcMAF): isoelectric focusing pattern and tumoricidal activity. Anticancer Res *23*: 4451-4458, 2003.

8 Uto Y, Yamamoto S, Takeuchi R, Nagasawa Y, Hirota K, Terada H, Onizuka S, Nakata E and Hori H: Effect of the Gc-derived macrophage-activating factor precursor (preGcMAF) on phagocytic activation of mouse peritoneal macrophages. Anticancer Res *31*: 2489-2492, 2011.

9 Cannon GJ and Swanson A: The macrophage capacity for phagocytosis. J Cell Sci *101*: 907-913, 1992.

10 Kadowaki T, Inagawa H, Kohchi C, Hirashima M and Soma G: Functional characterization of lipopolysaccharide derived from symbiotic bacteria in rice as a macrophage-activating substance. Anticancer Res *31*: 2467-2476, 2011.

11 Takasaki S, Emling F and Leive L: Variants deficient in phagocytosis of latex beads isolated from the murine macrophage-like cell line J774. J Cell Biol *98*: 2198-2203, 1984.

12 Gopinath VK, Musa M, Samsudin AR, Lalitha P and Sosroseno W: Role of nitric oxide in hydroxyapatite-induced phagocytosis by murine macrophage cell line (RAW264.7). Arch Oral Biol *51*: 339-344, 2006.

13 Kuchiike D, Uto Y, Mukai H, Ishiyama N, Abe C, Tanaka D, Kawai T, Kubo K, Mette M, Inui T, Endo Y and Hori H: Degalactosylated/desialylated human serum containing GcMAF Induces macrophage phagocytic activity and *in vitro* antitumor activity. Anticancer Res *33*: 2881-2886, 2013.

14 Hass R, Bartels H, Topley N, Hadam M, Kohler L, Goppelt-Strube M and Resch K: TPA-induced differentiation and adhesion of U937 cells: changes in ultrastructure, cytoskeletal organization and expression of cell surface antigens. Eur J Cell Biol *48*: 282-293, 1989.

15 Morganthaler JJ and Price CA: Density-gradient sedimentation in silica sols. Anomalous shifts in the banding densities of polystyrene "latex" beads. J Biochem *153*: 487-490, 1976.

16 Kobayashi S, Kojidani T, Osakada H, Yamamoto A, Yoshimori T, Hiraoka Y and Haraguchi T: Artifical induction of autophagy around polystyrene beads in nonphagocytic cells. Autophagy *6*: 1-10, 2010.

Received April 4, 2014
Revised June 7, 2014
Accepted June 10, 2014