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# Ubiquitin-like protein MNSFβ regulates glycolysis and promotes cell proliferation with HSC70 assistance

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## ARTICLE INFO

## ABSTRACT

Monoclonal non-specific suppressor factor β (MNSFβ) is a universally expressed ubiquitin-like protein that has multiple biological functions. MNSFβ modifies its target molecules through covalent conjugation. Most recently, we identified a molecular chaperone, HSC70, that facilitates the stabilization of aggregable MNSFβ. In the current study, we determined the role of HSC70 in stabilizing unstable MNSFβ. HSC70 promoted the correct folding of MNSFβ both *in vitro* and *in vivo*. We also examined the regulatory function of MNSFβ in cell proliferation and glycolysis. MNSFβ siRNA and HSC70 siRNA treatment attenuated lactate release from Raw264.7 macrophage-like cells. MNSFβ siRNA inhibited glucose uptake in Raw264.7 cells. We found that glucose transporter 1 (GLUT1) is an important membrane protein involved in the regulatory function of MNSFβ during glycolysis. MNSFβ siRNA inhibited the increased GLUT1 expression in LPS-stimulated cells, suggesting that MNSFβ controls the inflammatory response through GLUT1 regulation. We identified several important molecules, including lactate dehydrogenase A, which are regulated by MNSFβ and involved in glucose metabolism. Here we firstly report that MNSFβ regulates glycolysis and promotes cell proliferation.

## 1. Introduction

Monoclonal nonspecific suppressor factor (MNSF), a cytokine produced by mouse T cell hybridomas, exhibits nonspecific antigen inhibitory functions [1]. We have cloned a cDNA for a subunit of MNSF, which was designated as MNSFβ [2]. The MNSFβ cDNA encodes a fusion protein consisting of a ubiquitin-like domain (74 amino acids) and ribosomal protein S30 (59 amino acids). The ubiquitin-like domain exhibits MNSF-like biological activity [1]. Interferon-γ (IFNγ) is involved in the mechanism of action of MNSFβ [3]. We reported that MNSFβ specifically binds to cell-surface receptors on mitogen-triggered lymphocytes and mouse D.10 cells, which are T helper type 2 (Th2) cells [4]. However, because of the high similarity of MNSFβ with ubiquitin (36%) and the preservation of the Gly–Gly doublet at the C-terminus of the ubiquitin-like domain, which is necessary for ubiquitination, we have focused our study on the intracellular activity of MNSFβ. Post-translational modification by ubiquitin-like proteins regulates a variety of eukaryotic processes [5,6]. We demonstrated that MNSFβ covalently ligates to various target molecules and forms MNSFβ adducts in IFNγ- and concanavalin A-activated D.10 cells. The 33.5-kDa MNSFβ adduct consists of the proapoptotic protein Bcl-G and MNSFβ [3]. Unlike

ubiquitin, MNSFβ is not involved in proteolysis; however, MNSFβ regulates a variety of biological processes, including apoptosis, cell division, immunomodulation and stress regulation. We found that the Bcl-G/MNSFβ complex associates with ERKs and regulates the ERK-/MAPK signal pathway, which results in RANTES (regulated on activation, normal T cell expressed and secreted) and TNFα production in the murine macrophage-like cell line Raw264.7 [7]. MNSFβ covalently ligates to Bcl-G and enhances IFNγ/LPS-induced apoptosis in Raw264.7 cells [8]. In addition, cytosolic 10-formyltetrahydrofolate dehydrogenase is covalently attached to MNSFβ and controls thymocyte apoptosis [9]. We recently demonstrated that MNSFβ noncovalently attaches to HSC70/HSPA8 and controls osteoclastogenesis [10]. HSC70 plays a key role in the protein quality management system [11–14]. Double knockdown of HSC70 and MNSFβ strongly inhibited receptor activator of NF-κB ligand-induced osteoclastogenesis in Raw264.7 cells, suggesting that the association of HSC70 with MNSFβ facilitates this osteoclastogenic process [10]. To the best of our knowledge, this was the first study showing that MNSFβ noncovalently binds to a protein target to exert a biological function and indicates that HSC70 supports the proper folding of aggregable MNSFβ.

In the present study, we further verified this chaperone mechanism

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and examined the regulatory mechanism of cell proliferation through MNSFβ in the context of glucose metabolism. Glucose transporters (GLUTs) belong to the *Slc2a* gene family [15,16]. Most studies regarding macrophage function have focused on the action of GLUT1 and GLUT3 [17,18]. We also found that GLUT1 is an important GLUT involved in the regulatory effect of MNSFβ on glucose metabolism and cell proliferation.

## 2. Materials and methods

### 2.1. Antibodies and chemicals

Rabbit anti-MNSFβ antibodies were prepared as described previously [2]. Rabbit polyclonal antibodies against HSC70 and phospho-AMPKα1 (Ser485) and rabbit monoclonal antibodies against PKM2 and PFKFB3 were obtained from Cell Signaling Technology. Rabbit anti-GLUT1 and anti-GLUT3 antibodies were purchased from Proteintech and Abcam, respectively. Rabbit polyclonal antibodies against PDK1 and LDHA were obtained from ABclonal. Phospho-AKT (Ser473) rabbit polyclonal antibodies were obtained from GeneTex. Peroxidase-conjugated anti-rabbit IgG was purchased from Cappel (Solon, OH, USA). LPS (*Escherichia coli* 026:B6) was purchased from Sigma.

### 2.2. Western blot analysis

Western blot analysis was performed as previously described [7]. The cells were harvested, boiled in SDS lysis buffer, and resolved by gel electrophoresis. The blotted proteins were transferred onto polyvinylidene fluoride membranes, and the membranes were blocked overnight at 4 °C in Tris-buffered saline (TBS) containing 5% non-fat dry milk (NFDM). They were incubated with the primary antibodies for 2 h at 25 °C in TBS containing 0.1% Tween-20 (Tris/Tween). This step was followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 2 h at 25 °C in Tris/Tween with 5% NFDM. The labeled proteins were visualized by chemiluminescence according to the manufacturer's protocols (Amersham Biosciences).

### 2.3. Cell culture, siRNAs, and transfection of cells

The mouse monocyte/macrophage-like cell line, Raw264.7 (ATCC TIB-71), was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma) and penicillin/streptomycin (100 U/ml and 100 μg/ml) at 37 °C in a 5% $CO_2$ atmosphere. siRNAs were purchased from Qiagen (Chatsworth, CA, USA). The target sequences were as follows: HSC70 siRNA (5′-AAGGTCGGAGCT-GAAAGGAAT-3′) and MNSFβ siRNA (5′-CCACCCTGCCATGCTAATAAA-3′). Scrambled control siRNA directed to 5′-GGACTCGACG-CAATGGCGTCA-3′ was used as a negative control. The cells were transfected with siRNAs (30 nM) using the HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's protocols. Raw264.7 cells ($5 \times 10^4$) were transfected with siRNA directed against MNSFβ or HSC70 as previously described [10]. Complementary DNA encoding MNSFβ and HSC70 were sub-cloned into the vector pcDNA3.1(+) (Invitrogen) vector, as described previously [10].

### 2.4. Cloning, transfection and confocal imaging of live cells

Human MNSFβ (GenBank: CAA46714.1) was inserted into the pAcGFP1-C1 vector (Clontech), and human HSC70 (GenBank: ADE34170) was inserted into the pmCherry-C1 vector (Clontech) by Ligation high (TOYOBO). Human epithelial cell line, HeLa (ATCC CCL-2), was transfected using lipofectamine 2000 (Invitrogen). About $5 \times 10^4$ HeLa cells were grown in each well of a 24-well plate. The transfection was performed with 2 μg plasmid and 4 μg lipofectamine 2000 in 200 μl Opti-MEM (GIBCO). Fluorescence signals were detected using a laser scanning confocal microscope (FV1000D, Olympus, Tokyo Japan).

### 2.5. Glucose and lactate assay

Cells were seeded at a density of $1 \times 10^5$ cells/ml in each well of a 24-well plate. To measure the glucose concentration in the culture supernatant, we used a glucose assay kit (WST; Dojindo) based on the manufacturer's instructions. Glucose concentrations in the culture medium were calculated using a calibration curve generated from the glucose standards. To calculate glucose consumption per cell, the glucose concentration after culturing was subtracted from the glucose concentration of the culture broth, and the concentration was normalized to the number of cells. Using a glycolysis cell-based assay kit (Cayman Chemical), lactic acid concentrations were calculated using a lactic acid standard and normalized to cell number.

### 2.6. GST-MNSFβ affinity chromatography and protein aggregation assay

GST-MNSFβ recombinant protein (1 μg) immobilized on 15 μl of glutathione Sepharose 4B resin was incubated with 600 μg of lysates of *E. coli* transformed with GFP-HSC70 cDNA, as described previously [10]. The reacted resin was then washed and incubated with or without thrombin for 12 h at 22 °C. The reacted resin was washed, and the wash buffer was collected and used for the protein aggregation assay described below. Protein aggregation was qualitatively measured using a ProteoStat® protein aggregation assay kit according to the manufacturer's instructions (Enzo Life Sciences). Briefly, samples obtained by affinity chromatography as described above were mixed well with ProteoStat dye and incubated for 15 min at 25 °C. The reaction intensity was measured with a microplate reader (Bio-Rad).

### 2.7. Statistical analysis

Statistical significance was analyzed using a Student's *t*-test and expressed as *p* values. A *p* value of 0.05 or less was considered statistically significant.

## 3. Results

### 3.1. The ubiquitin-like protein MNSFβ promotes cell proliferation in the Raw264.7 mouse macrophage-like cell line

The mode of function of MNSFβ has been studied exhaustively in our laboratory; however it is unclear how MNSFβ regulates cell proliferation. Therefore, we examined the proliferation of Raw264.7 cells studied in our laboratory, which were treated with 30 nM MNSFβ siRNA for 3 days. The experiments were designed to avoid the over-growth of the cells. As shown in Fig. 1A, the proliferation of Raw264.7 cells treated with MNSFβ siRNA was strongly inhibited compared with that of the cells treated with control siRNA. Recently, we reported that the molecular chaperone, HSC70, assists in the folding of the very aggregable MNSFβ [2]. Raw264.7 cells were treated with HSC70 siRNA, and cell proliferation was determined. Similar to MNSFβ siRNA, HSC70 siRNA also inhibited cell proliferation (Fig. 1A). Co-transfection of both siRNAs against MNSFβ and HSC70 markedly decreased the cell proliferation. Silencing of MNSFβ did not induce cell death in Raw264.7 cells as previously described [8]. RT-PCR analysis revealed that MNSFβ siRNA and HSC70 siRNA specifically reduced the expression of MNSFβ and HSC70, respectively (Fig. 1C). These observations strongly suggest that HSC70 promotes the correct folding of aggregative MNSFβ, as previously described [10].

### 3.2. HSC70 facilitates the proper folding of aggregable MNSFβ in vitro and in vivo

Raw264.7 cells treated with MNSFβ siRNA and HSC70 siRNA were analyzed by western blotting. As previously reported [3,7–9], MNSFβ conjugates to various target proteins (Fig. 2 A, lane 1). The MNSFβ
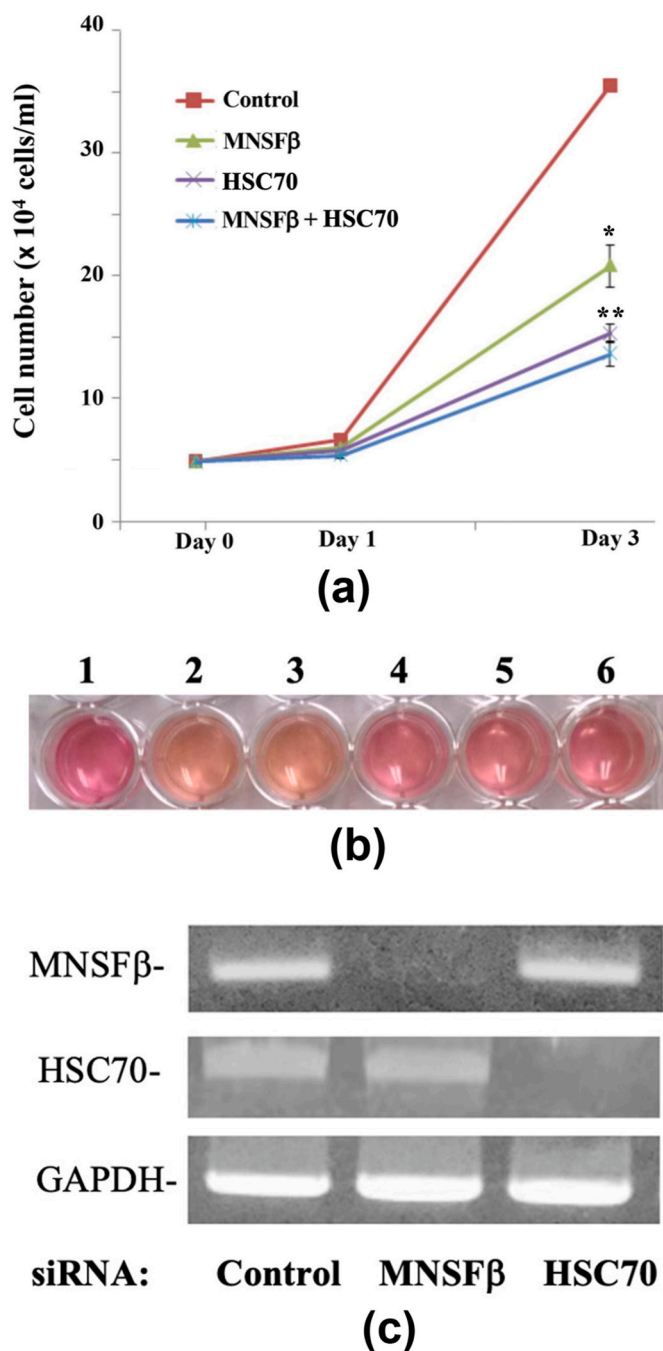
**Fig. 1.** MNSFβ enhances cell proliferation and HSC70 promotes MNSFβ adduct formation. (A) MNSFβ and HSC70 siRNAs inhibit the proliferation of Raw264.7 cells. Cells were transfected with siRNA directed against MNSFβ, HSC70 or scrambled siRNA. The number of cells was counted over time for 3 days. Except for siRNA treatment, cells were cultured without any stimulation. *$p < 0.05$ versus cells treated with scrambled siRNA; **$p < 0.01$ versus cells treated with scrambled siRNA (B) Raw264.7 cells were transfected with siRNA directed against MNSFβ, HSC70, or scrambled siRNA. After 72 h of transfection, images of the culture medium were captured. Lane 1, medium; lane 2, no siRNA; lane 3, control scramble siRNA; lane 4; MNSFβ siRNA; lane 5, HSC70 siRNA; lane 6, MNSFβ siRNA plus HSC70 siRNA. (C) MNSFβ and HSC70 mRNA expression was analyzed by RT-PCR after treatment with each siRNA for 48 h.

adduct formation was decreased in both MNSFβ siRNA and HSC70 siRNA transfected cells (Fig. 2A, lanes 2 and 3). HSC70 greatly affected MNSFβ aggregation and adduct formation. Raw264.7 cells were also transfected with MNSFβ cDNA and HSC70 cDNA and analyzed by

western blotting. As expected, the MNSFβ adduct formation was increased in both MNSFβ cDNA and HSC70 cDNA transfected cells (Fig. 2B, lanes 2 and 3).

To directly prove the chaperone function of HSC70, we performed a protein aggregation assay using ProteoStat®, a protein agglutination detection reagent, to examine the inhibition of MNSFβ aggregation. MNSFβ is an aggregable polypeptide, and even recombinant MNSFβ tends to form aggregates [2]. As shown in Fig. 2C, free 8.5 kDa-MNSFβ was not observed (lane 4). Because the GST-MNSFβ fusion recombinant protein is very stable, we used this fusion protein for the aggregation assay. The MNSFβ segment can be cleaved from its GST fusion partner by thrombin [2]. GST-MNSFβ affinity chromatography was performed with GFP-HSC70 in the presence or absence of ATP as described in Materials and Methods. After thrombin digestion, the effect of HSC70 on MNSFβ disaggregation was examined by using ProteoStat®. As shown in Fig. 2D, GFP-HSC70 significantly inhibited MNSFβ aggregation compared with the control assay without GFP-HSC70. MNSFβ aggregation was not observed in the absence of thrombin (Fig. 2D). To prove MNSFβ and HSC70 are in the vicinity of the cells at some point, we co-transfected HeLa cells with plasmids carrying GFP-MNSFβ and mCherry-HSC70. Fig. 2E displays typical localization of the fluorescently labeled proteins in live-cells. MNSFβ predominantly localized in the nucleolus and HSC70 existed throughout the cells. The results demonstrate that MNSFβ and HSC70 are co-localized in the nucleus, although the signal in the merged images was weak.

### 3.3. MNSFβ siRNA inhibits both lactate release and glucose uptake in Raw264.7 cells

No difference was observed in the color of the culture medium (purple-pink coloration/yellow coloration) between the cells treated with MNSFβ siRNA and those treated with control (scramble) siRNA (Fig. 1B). The change in the color of the medium indicates that the pH has changed because of the release of lactate by Raw264.7 cells. To confirm this effect, we measured the amount of lactic acid secreted by Raw264.7 cells. The measured amount of lactic acid was adjusted according by cell number because MNSFβ siRNA-treated cells suppress proliferation, as described above. The secretion of lactic acid was strongly inhibited by the treatment with MNSFβ siRNA (Fig. 3A). Similarly, lactic acid release was inhibited in HSC70 siRNA transfected Raw264.7 cells. Double knockdown of MNSFβ and HSC70 was not significantly different from that of single siRNA treatment. These results support our hypothesis that HSC70 is an important chaperone that stabilizes the activity of MNSFβ. Next, we determined whether MNSFβ siRNA treatment affects lactic acid release by LPS-stimulated Raw264.7 cells. Lactic acid release was increased by LPS stimulation in Raw264.7 cells (Fig. 3B), and this increase of lactic acid was significantly inhibited by the treatment with MNSFβ siRNA (Fig. 3B). Lactic acid release and glucose uptake are closely involved in glycolysis. To examine whether MNSFβ siRNA affects glucose uptake, we used a glucose assay system based on a colorimetric WST reaction. The results indicated that MNSFβ siRNA treatment had a significant inhibitory effect on glucose uptake in unstimulated Raw264.7 cells (Fig. 3C, lane 3). Similar to MNSFβ siRNA, HSC70 siRNA also significantly inhibited the glucose uptake (Fig. 3C, lane 4).

### 3.4. MNSFβ regulates GLUT1 expression and key molecules in glucose metabolism in Raw264.7 cells

Based on the results shown in Fig. 3, we evaluated GLUT expression in Raw264.7 cells. Ahmed et al. showed that the main GLUT isoform expressed in Raw264.7 cells is GLUT3 [19]; however, Freemerman et al. and other groups reported that GLUT1 mediates glucose metabolism in Raw264.7 cells [20,21]. Thus, we first identified the major GLUT that regulates glucose metabolism in Raw264.7 cells. As shown in Fig. 4A, strong expression of GLUT1 was observed in whole cells, and both
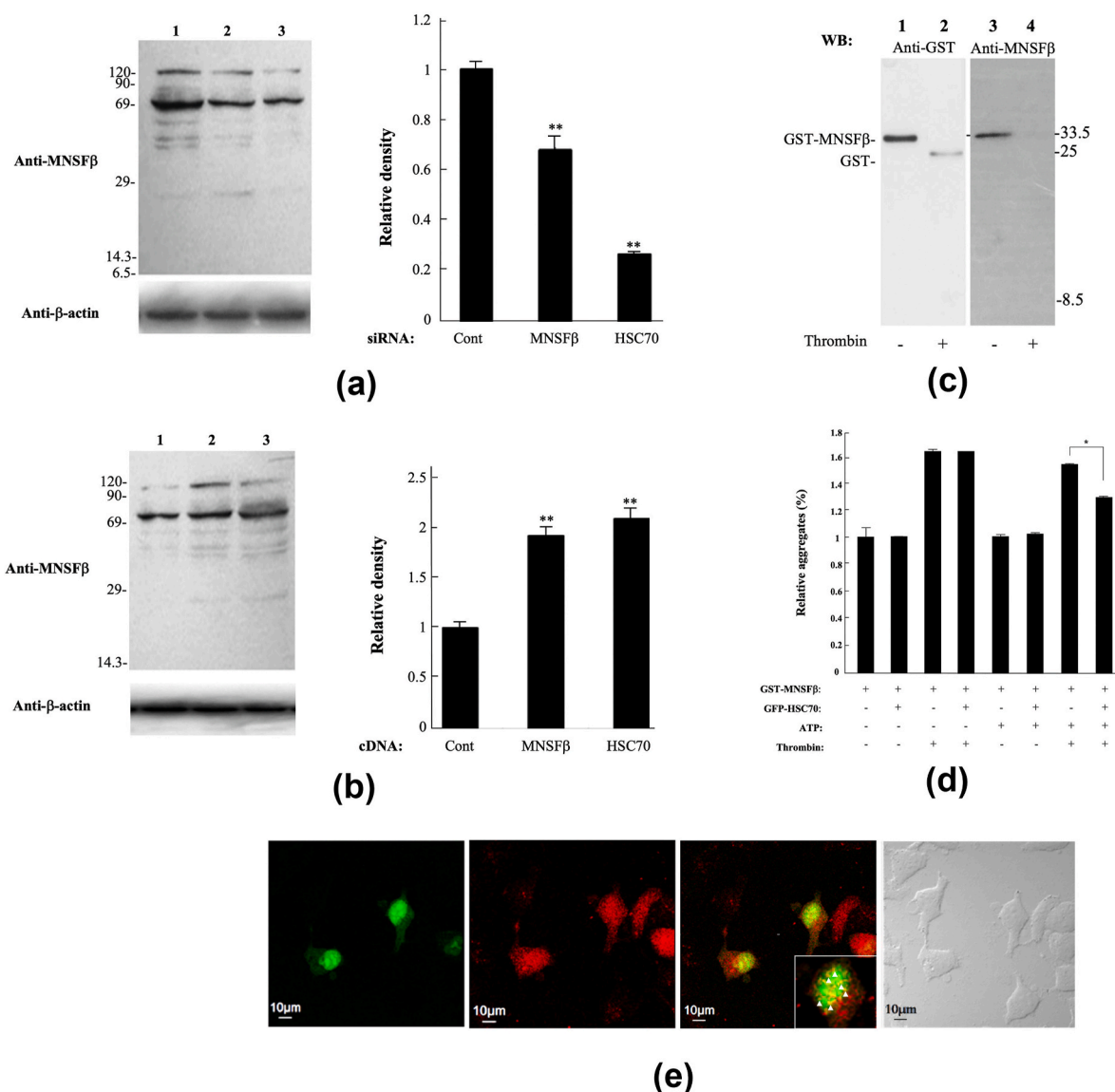
**Fig. 2.** HSC70 promotes correct folding of MNSFβ *in vitro* and *in vivo* experiments. (A) Raw264.7 cells were transfected with siRNA directed against MNSFβ, HSC70, or scrambled siRNA. After 48 h of siRNA transfection, cell extracts were subjected to immunoblot analysis with anti-MNSFβ Ab. The presence of MNSFβ-adducts in the cell lysates was detected by Western blot analysis. The intensity of the total signal was determined by densitometric scanning and expressed as the fold-change relative to that of untreated cells. Values are expressed as mean ± SD (n = 3). A representative autoradiograph is shown. A molecular mass standard (kDa) is indicated to the left. Lane 1, control siRNA; lane 2, MNSFβ siRNA; lane 3, HSC70 siRNA. Asterisks (**) indicate statistically significant differences (*p* < 0.01) versus the controls. (B) Raw264.7 cells were transfected with MNSFβ cDNA and HSC70 cDNA. After 48 h of cDNA transfection, cell extracts were subjected to immunoblot analysis with anti-MNSFβ Ab. The presence of MNSFβ-adducts in the cell lysates was analyzed described above. Lane 1, control cDNA; lane 2, MNSFβ cDNA; lane 3, HSC70 cDNA. Values are means ± SD (n = 3). A representative autoradiograph is shown. Asterisks indicate statistically significant differences (*p* < 0.01) versus the controls. (C) GST-MNSFβ affinity chromatography was performed as described in Materials and methods section. GST-MNSFβ immobilized on glutathione Sepharose 4B resin was treated with thrombin (lanes 2 and 4) and eluted with glutathione, and the eluant was immunoblotted with anti-GST (lanes 1 and 2) and anti-MNSFβ (lanes 3 and 4) antibodies. (D) GST-MNSFβ and GFP-HSC70 were combined, and protein aggregation (aggregated MNSFβ) was qualitatively measured using a ProteoStat® protein aggregation assay kit as described in the Materials and methods section. GFP-HSC70 stabilized free unstable MNSFβ. Values are expressed as the mean ± standard deviation of triplicate samples. *p* < 0.05 versus without GFP-HSC70. (E) HeLa cells were co-transfected with plasmids carrying GFP-MNSFβ and mCherry-HSC70 as described in the Materials and Methods section. Fluorescence signals were detected using a laser scanning confocal microscope. Bottom right of the merged photo, a close-up-photograph of the co-localization; white arrow heads show the co-localization of MNSFβ with HSC70.

MNSFβ and HSC70 siRNAs potently inhibited the GLUT1 expression. Next, the cells were fractionated into membrane and cytoplasm fractions, and the expression of each GLUT1 and GLUT3 was analyzed by Western blot analysis. F4/80 was used as a membrane marker. Fig. 4B clearly shows that the cells were correctly fractionated as GLUT1 was strongly expressed in the membrane, but this transporter was not detectable in the cytoplasm. In contrast, GLUT3 was strongly expressed in the cytoplasm, but was not translocated to the cell membrane of unstimulated Raw264.7 cells. Collectively, GLUT1 mediates glucose metabolism in macrophages, and MNSFβ regulates the cell membrane expression of GLUT1. In addition, MNSFβ may not be involved in the translocation of GLUT1 to the cell membrane because GLUT1 was not observed in the cytosol of unstimulated cells (Fig. 4B). It was previously reported that GLUT1 expression was increased in LPS-stimulated Raw264.7 cells [22,23]. We examined whether MNSFβ siRNA affects the GLUT1 increase in the LPS (1 μg/ml)-stimulated cells. As shown in Fig. 4C (lane 4), the cell membrane expression of GLUT1 was increased, and its expression in the cytosol was slightly detectable. MNSFβ siRNA
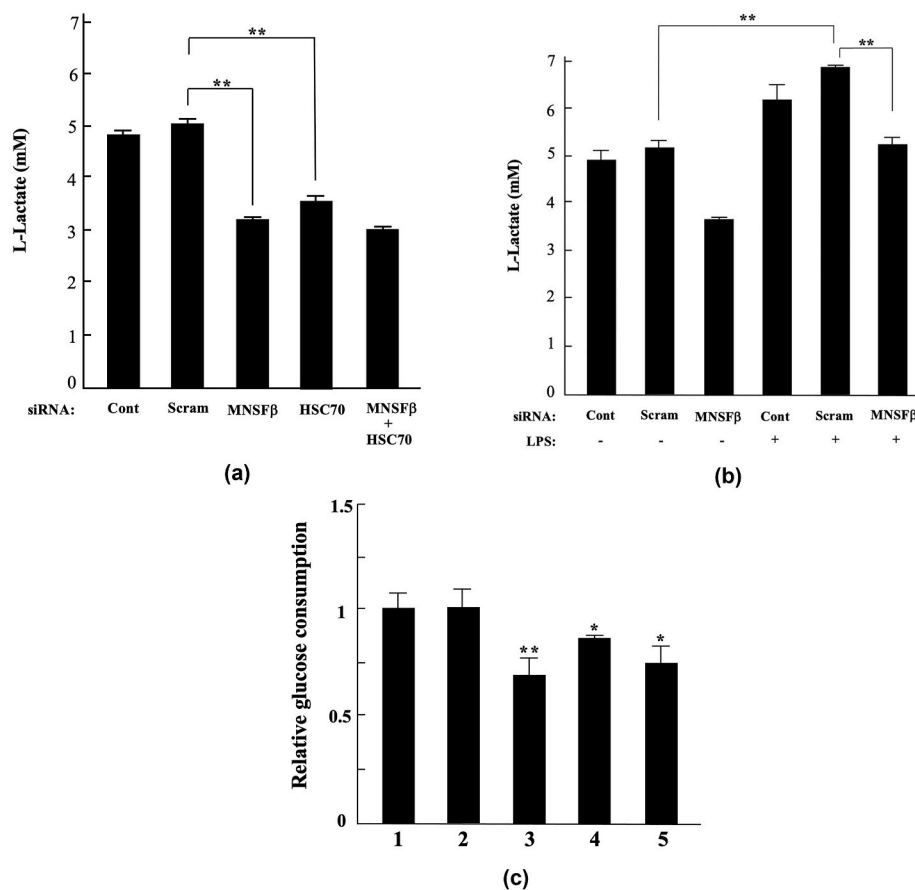
**Fig. 3.** MNSFβ siRNA inhibits lactate release and glucose uptake in Raw264.7 cells. (A) The amount of lactate in the culture medium of cells treated with MNSFβ siRNA was measured using a lactate assay kit as described in the Materials and methods section. The amount of lactate released from the cells treated with HSC70 siRNA was also measured. **$p < 0.01$ versus cells treated with scrambled siRNA (B) The effect of MNSFβ siRNA on increased lactate release in LPS-stimulated cells was determined. **$p < 0.01$ versus cells treated with scrambled siRNA with or without LPS (C) The uptake of glucose by Raw264.7 cells was tested using a glucose assay kit-WST as described in the Materials and methods section. Lane 1, control (no siRNA); lane 2, scramble siRNA; lane 3, MNSFβ siRNA; lane 4, HSC70 siRNA; lane 5, MNSFβ siRNA plus HSC70 siRNA. *$p < 0.05$ versus cells treated with scrambled siRNA; **$p < 0.01$ versus cells treated with scrambled siRNA.

treatment inhibited the increase of both in LPS-stimulated cells. We did not observe the cell membrane expression of GLUT3 in LPS-stimulated cells (Fig. 4, lane 3). GLUT3 expression was slightly decreased in Raw264.7 cells treated with MNSFβ siRNA (Fig. 4, lanes 6 and 8). Finally, we identified the key molecules in glucose metabolism regulated by MNSFβ. As shown in Fig. 4D, the expression of pyruvate dehydrogenase kinase 1 (PDK1), pyruvate kinase isozyme M2 (PKM2), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), and lactate dehydrogenase A (LDHA) was significantly inhibited in the Raw264.7 cells treated with MNSFβ siRNA. The phosphorylation (activation) of AKT, but not of AMP-activated protein kinase α1 (AMPKα1), was also inhibited. Therefore, MNSFβ regulates several important molecules involved in glycolysis.

## 4. Discussion

We have studied the role of MNSFβ in various processes, including apoptosis, phagocytosis, and cell differentiation [7–10]. In the present study, we focused on the mechanism of the action of MNSFβ with respect to cell proliferation. The motivation for this study was based upon two observations; 1) the proliferation of the cells treated with MNSFβ siRNA was suppressed; and 2) the change of color in the culture medium (purple-pink coloration/yellow coloration) was quite different between MNSFβ siRNA-treated and untreated cells, which suggests a difference in lactate secretion. A weak synergistic effect of MNSFβ and HSC70 siRNAs was observed in the cell proliferation experiments; however, a synergistic effect was not confirmed with respect to lactate secretion (Fig. 1). An association of glycolysis and HSC70 has been reported in 24 types of cancer [24]. During glycolysis and cell proliferation, HSC70 may have other function(s) in addition to the function, described below, specialized for the chaperone effect on MNSFβ.

The HSC70 molecular chaperone is important for the function of MNSFβ. HSC70 assists with the correct folding of the very aggregable MNSFβ [10]. In the present study, we directly demonstrated the chaperone function of HSC70 through the use of a stable recombinant GST-MNSFβ fusion protein. We confirmed that HSC70 suppresses the aggregation of MNSFβ with protein aggregation assay (Fig. 2). MNSFβ readily aggregates after cleavage by thrombin, and the 8.5 kDa-MNSFβ product was not observed because of the formation of high molecular weight aggregates [2]. For this experiment, we used a GFP-HSC70 fusion protein, which is frequently used for fluorescence experiments (manuscript in preparation). GFP-HSC70 properly exerted the chaperone function in Raw264.7 cells (data not shown). Using both MNSFβ and HSC70 siRNAs and MNSFβ and HSC70 cDNAs, the results indicated that HSC70 exhibits a chaperone effect on MNSFβ-adduct formation (Fig. 2). Confocal imaging of live cells indicates that MNSFβ exhibits very weak co-localization with HSC70 (Fig. 2). We have previously reported that MNSFβ (ubiquitin-like segment) is expressed as a fusion protein with the ribosomal protein S30 at its terminus [2]. Although the fusion protein is very stable, the cleavage of MNSFβ from ribosomal protein S30 intracellularly is highly unstable and aggregated. In this study, only a part of MNSFβ was expressed in live cells. It is currently not known how HSC70 is involved in this fusion protein for binding of the stabilized MNSFβ to its target proteins. The fact that MNSFβ is produced as a fusion protein may affect the confocal imaging of live cells.

Because of the difference in lactate levels in the cell culture medium, with or without MNSFβ siRNA treatment, we examined the function of MNSFβ in glycolysis. We focused on glucose GLUTs because they are the important components of the cellular glucose uptake system. The *SLC2* (SoLute Carrier) genes code for a family of GLUT proteins, and the GLUTs1–4, GLUT5, and GLUT13 are well-characterized GLUT proteins [16]. The major GLUT protein expressed in Raw264.7 cells is

(a)

(b)

(c)

(d)
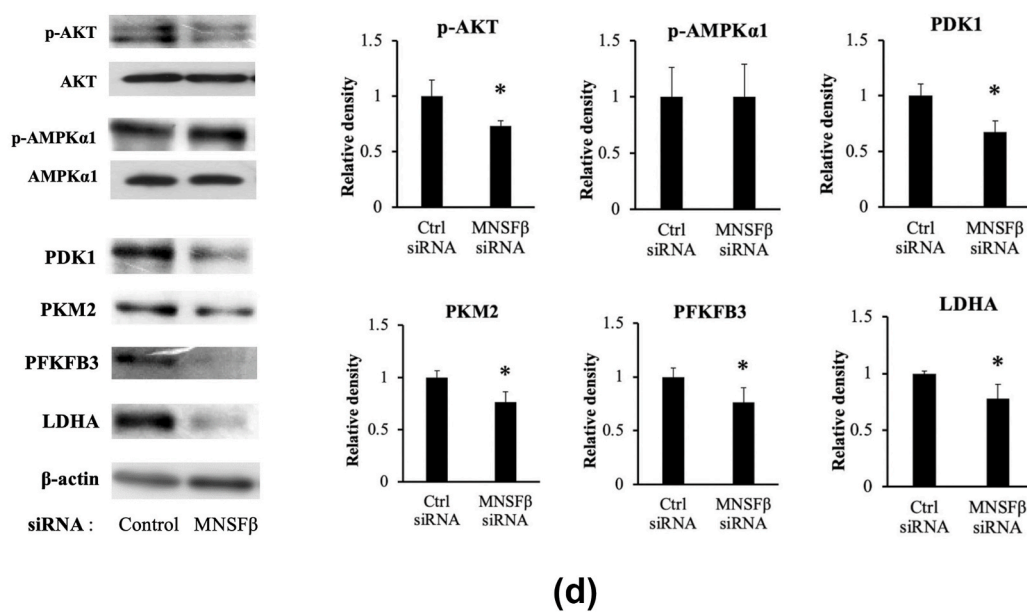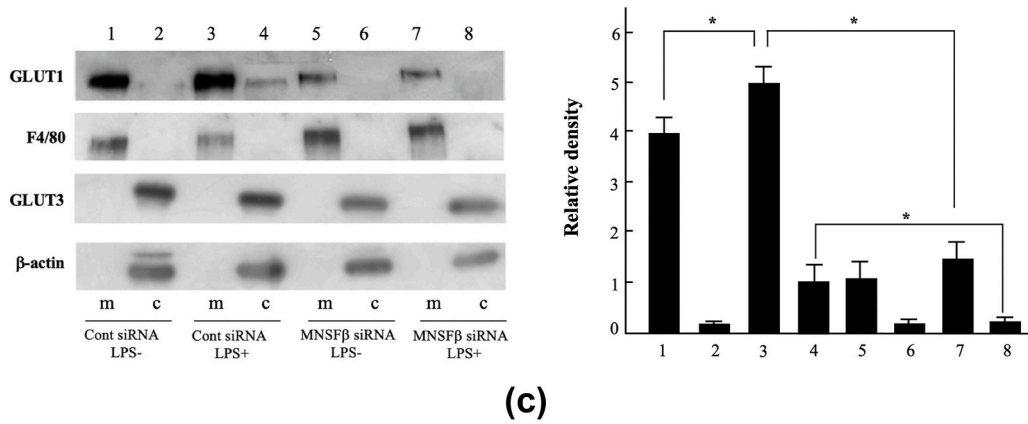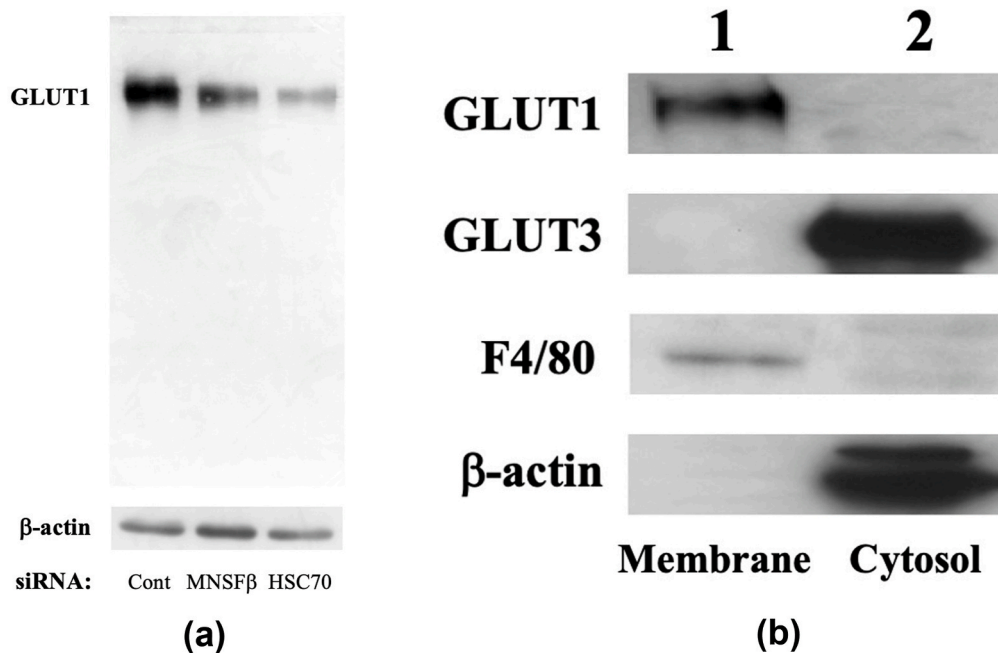
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**Fig. 4.** GLUT1 is a key factor involved in the regulatory activity of MNSFβ. (A) GLUT1 expression in unstimulated Raw264.7 cells treated with MNSFβ siRNA or HSC70 siRNA was determined by Western blot analysis using anti-GLUT1 antibody. (B) The cells were fractionated into the membrane and cytosol, and a Western blot assay was performed using anti-GLUT1 and anti-GLUT3 antibodies for each fraction. m, membrane fraction; c, cytosol. F4/80 was employed as a membrane marker. (C) The effect of MNSFβ siRNA on GLUT1 and GLUT3 expression was evaluated in LPS-stimulated cells. Values are expressed as mean ± standard deviation of triplicate samples. $*p < 0.05$ versus cells not treated with LPS or cells not treated MNSFβ siRNA (D) Western blot analysis was performed on several molecules involved in glucose metabolism. *p*-AKT, phosphorylated AKT; *p*-AMPKα1, phosphorylated AMPKα1. Values are expressed as mean ± standard deviation of triplicate samples. $*p < 0.05$ versus cells not treated with MNSFβ siRNA.

controversial, as described in the Results section. We confirmed that GLUT1 is constitutively and strongly expressed in unstimulated Raw264.7 cells (Fig. 4A). We found that GLUT1 is also strongly expressed in the membrane of unstimulated Raw264.7 cells (Fig. 4B). The GLUT1 expression in the membrane was strongly inhibited in the cells treated with MNSFβ siRNA (Fig. 4C). Based on our results and those of others, GLUT1 primarily mediates glucose uptake and glycolytic metabolism in macrophages [20,21] and MNSFβ is involved in the glycolytic metabolism. We identified several key molecules in glucose metabolism regulated by MNSFβ. Of note, MNSFβ regulates LDHA (Fig. 4D), which is directly involved in lactate production. PFKFB3 is a critical regulatory enzyme in glycolysis control [25]. PFKFB3 and GLUT1 are closely regulated by HIF-1α in various cell lines, including epithelial cells [26]. Recently, we observed that MNSFβ directly controls HIF-1α expression in Raw264.6 cells (manuscript in preparation). Further investigation is ongoing in our laboratory to clarify the mechanism of action of MNSFβ in glycolysis.

Reprogramming during macrophage activation closely resembles the characteristics of cancer cells [27]. When stimulated with various cytokines or LPS, resting macrophages differentiate into M1 macrophages (pro-inflammatory phenotype). LPS-triggered macrophages show metabolic changes that result in the upregulation of glycolysis and increased lactate release (known as aerobic glycolysis) [23,28]. We have demonstrated that MNSFβ regulates inflammatory cytokine production including LPS-activated macrophages [7]. In the present study, we showed that MNSFβ regulates GLUT1 expression in the cell membrane (Fig. 4). Therefore, the regulation of inflammatory cytokine production by MNSFβ may be due, in part, to the regulation of GLUT1 expression. HIF-1α, which stabilizes MNSFβ, may be also involved in GLUT1 regulation.

Yang et al. reported that lactate inhibits pro-inflammatory responses in Raw264.7 cells [29]. In this context, a lactate autocrine mechanism regulated by MNSFβ should be investigated. Further studies are needed to clarify the regulatory effect of MNSFβ on the inflammatory response.

## Author contributions

M.N. conceived the study. M.N., K.Y., and M.K. conducted the experiments. M.N. and M.K. performed the statistical analysis. M.N. and M. K. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

## Declarations of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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