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GDF1 is a novel mediator of macrophage infiltration in brown adipose tissue of obese mice

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ABSTRACT

We previously demonstrated a marked upregulation in the bone morphogenic protein (BMP)/growth differentiation factor (GDF) family member, GDF5, which is capable of promoting brown adipogenesis, in brown adipose tissue (BAT) of obese mice. In this study, we identified other GDF family members, besides GDF5 that are responsive to different obesogenic signals in BAT using inborn and acquired obesity animal models. In BAT from leptin-deficient *ob/ob* mice, *GDF1* expression was preferentially downregulated, whereas the expression of several other genes in the BMP/GDF family, including GDF5, was upregulated. Moreover, in cultured brown adipocytes exposed to tunicamycin and hydrogen peroxide, at concentrations not affecting cellular viability, *GDF1* expression was significantly downregulated. Recombinant GDF1 failed to significantly alter brown adipogenesis, despite the promoted phosphorylation of Smad1/5/8 in cultured brown adipocytes, but accelerated Smad1/5/8 phosphorylation with a concomitant increase in the number of migrating cells during exposure in a manner sensitive to activin-like kinase inhibitors in macrophagic RAW264.7 cells. Similarly, accelerated migration was observed in murine peritoneal macrophages exposed to GDF1. These results indicate that obesity could lead to predominant downregulation of GDF1 expression in BAT, which can modulate cellular migration through a mechanism relevant to activation of the downstream Smad signaling pathway in adjacent macrophages.

1. Introduction

Brown adipose tissue (BAT) generates heat through mitochondrial uncoupling of lipid oxidation, whereas white adipose tissue (WAT) serves as a storage depot for excess energy [1,2]. Recent studies have demonstrated that adult humans have substantial amounts of functioning BAT [3–6]. Accordingly, BAT presents a potential therapeutic target to combat obesity and related metabolic diseases through a mechanism relevant to accelerated energy expenditure in humans. Brown fat-like adipocytes, which have been described as brite or beige cells with a feature expressing uncoupling protein-1 (UCP1), are sporadically found in WAT, whereas classical brown adipocytes reside in the interscapular and perirenal regions.

Several factors are implicated in the development of beige cells in WAT. Of these, activation of peroxisome proliferator-activated receptor-γ (PPARγ) facilitates transformation of white adipocytes into brown adipocytes [7,8], whereas fibroblast growth factor-21 was found to promote the conversion of white adipocytes to brown adipocytes [9]. Bone morphogenic protein (BMP)-7 accelerates brown adipocyte differentiation and thermogenesis [10], whereas BMP8B increases BAT thermogenesis through both central and peripheral mechanisms [11]. In addition to these well-known endogenous factors, we recently reported the importance of growth differentiation factor-5 (GDF5), which is a member of the BMP/GDF family, in brown adipogenesis [12]. In fact, transgenic mice overexpressing GDF5 in adipose tissues display increased systemic energy expenditure along with the appearance of beige cells in subcutaneous WAT [12]. Taken together, these findings indicate that particular BMP/GDF family members may be implicated in the development and maturation of brown adipocytes. Thus, these previous findings prompted us to comprehensively search for particular BMP/GDF family members responsive to obesity in BAT using leptin-deficient *ob/ob* mice as well as obese mice fed with a high fat diet (HFD) *in vivo*.

Abbreviations: ALK, activin-like kinase; BAT, brown adipose tissue; BMP, bone morphogenic protein; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; ER, endoplasmic reticulum; GDF, growth differentiation factor; HFD, high fat diet; PCR, polymerase chain reaction; PPARγ, peroxisome proliferator-activated receptor-γ; UCP1, uncoupling protein-1; WAT, white adipose tissue; WT, wild-type

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2. Materials and methods

2.1. Materials

A brown adipocyte cell line derived from newborn wild-type (WT) mice was kindly provided by Dr. C.R. Kahn (Joslin Diabetes Center, Boston, MA, USA) [10]. Macrophagic RAW264.7 cells and adipocytic 3T3-L1 cells were obtained from ATCC (Manassas, VA, USA). Myoblastic C2C12 cells were provided by RIKEN (Tsukuba, Japan). Recombinant mouse GDF1 was purchased from R&D Systems (Minneapolis, MN, USA). Both LDN193189 and SB431542 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Anti-glyceraldehyde-3-phosphate dehydrogenase, anti-phospho Smad1/5/8, anti-phospho Smad2, and anti-phospho Akt were obtained from Cell Signaling Technology (Danvers, MA, USA). A Mouse GDF1 enzyme-linked immunosorbent assay (ELISA) Kit was obtained from Bluegene (Shanghai, China). Thunderbird SYBR qPCR Mix was supplied by Toyobo Co., Ltd. (Osaka, Japan) and specific primers for each gene for PCR were listed in Table 1. Diet-induced obesity (DIO) rodent purified diet with 60% energy from fat was obtained from Japan SLC (Shizuoka, Japan). Other chemicals used in this study were of the highest purity and commercially available.

2.2. Mice and ELISA

Male WT and *ob*/*ob* mice with a C57BL/6 background were obtained from Japan SLC (Shizuoka, Japan). The study was conducted in accordance with the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for the Ethical Use of Experimental Animals of Kanazawa University. Blood was collected from the heart of mice under anesthesia using needles and syringe, followed by leaving on ice for 5 min and subsequent centrifugation at 4 °C for 5 min at 20,000*g* for collection of plasma. BAT was removed from animals after decapitation, followed by rinsing with PBS and subsequent homogenization in PBS. The suspension was sonicated and then centrifugation at 4 °C for 5 min at 5000*g* for collection of supernatant. GDF1 level was measured by ELISA according to manufacturer's protocols.

2.3. Culturing of brown adipocytes, white adipocytes and myoblasts, Oil Red O staining, and determination of cellular viability

Brown pre-adipocyte cell lines were derived from newborn wild-type (WT) mice [10]. Brown pre-adipocytes were cultured in a differentiation induction cocktail (20 nM insulin, 1 nM triiodothyronine, 0.125 mM indomethacin, 0.5 μM dexamethasone,

Table 1
List of primers used for qPCR in this study.

Genes	Upstream (5'-3')	Downstream (5'-3')
Acvr1	TGCTAATGATGATGGCTTTCC	TTCACAGTGGTCCTCGTTCC
Acvrl1	GGGCCTTTTGATGCTGTCG	TGGCAGAATGGTCTCTTGCAG
Acvr1b	CCCCCTTGTTGTCCTCCT	GGCCCCATCTGTCTCACA
Acvr1c	GTCTGGCTCACCTGCACAT	CAGCTATGGCACAAGTGTCAC
Acvr2a	GCGTTCGCCGTCTTTCTTATC	GTTGGTTCTGTCTCTTTCCCAAT
Acvr2b	ACCCCCAGGTGTACTTCTG	CATGGCCGTAGGGAGGTTTC
Amh	CCACACCTCTCTCCACTGGTA	GGCACAAAGGTTCAGGGGG
ap2	GATGCCTTTGTGGGAACCT	CTGTCGTCTGCGGTGATTT
Arg1	GAACACGGCAGTGGCTTTAAC	TGCTTAGCTCTGTCTGCTTTGC
BMP1	TTGTACGCGAGAACATACAGC	CTGAGTCGGGTCCTTTGGC
BMP2	GGGACCCGCTGTCTTCTAGT	TCAACTCAAATTCGCTGAGGAC
BMP3	ACTCCGTGAGACTGAGCCAA	CCTGTCATAGAGCCACAGCATA
BMP4	TTCCTGGTAACCGAATGCTGA	CCTGAATCTCGGCGACTTTTT
BMP5	TTACTTAGGGGTATTGTGGGCT	CCGTCTCTCATGGTTCCGTAG
BMP6	AGAAGCGGGAGATGCAAAAGG	GACAGGGCGTTGTAGAGATCC
BMP7	ACGGACAGGGCTTCTCCTAC	ATGGTGGTATCGAGGGTGGAA
Bmpr1a	AATGCAAGGATTCACCGAAAGCCC	ACAGCCATGGAAATGAGCACAACC
Bmpr1b	CCCTCGGCCCAAGATCCTA	CAACAGGCATTCCAGAGTCATC
Bmpr2	AGCAATCGCCCATCGAGACTTGAA	TTCTGGAGGCATATAGCGCTTGGT
Cebpa	CAAGAACAGCAACGAGTACCG	GTCACTCGTCAACTCCAGCAC
Cebpb	CAAGTTCCGCAGGGTGCT	CCAAGAAGACGGTGGACAA
GDF1	TTCTGCCAGGGCACGTGCG	GGAGCAGCTGCGTGCATGAG
GDF2	CGCAGCCTTAACCTCAGC	GTTGGAGGCAGGCGTAGA
GDF3	ATGCAGCCTTATCAACGGCTT	AGGCGCTTTCTCTAATCCCAG
GDF5	ATCGGACTGTTCAACCTTTCAG	GCACTCTTATCAAGGGTTAGGTC
GDF6	TGCACGTGAACTTCAAGGAGCTGGGCT	TCATCAGCGTCTGGATGATGGCGTGGT
GDF7	GAGGGCGTTTGCGACTTTC	CTGCTTGTAGACCACGTTGTT
Id1	CGACTACATCAGGGACCTGCA	GAACACATGCCGCCTCGG
Inha	ATGCACAGGACCTCTGAACC	GGATGGCCGGAATACATAAG
Inhba	GGAGAACGGGTATGTGGAGA	TGGTCCTGGTTCTGTTAGCC
Inhbb	CCTGAGTGAATGCACACCAC	CGAGTCCAGTTTCGCCTAGT
Lefty1	TGTGTGTGCTCTTTGCTTCC	GGGGATTCTGTCCTTGGTTT
Mcp1	TCCCAATGAGTAGGCTGGAG	AAGTGCTTGAGGTGGTTGTG
Nodal	CCATGCCTACATCCAGAGCCTGC	TGGTGTTCCAGGAGGACCCTGCC
Pparg	TCAGCTCTGTGGACCTCTCC	ACCCTTGCATCCTTCACAAG
Ppargc1a	GTCAACAGCAAAAGCCACAA	TCTGGGGTCAGAGGAAGAGA
Prdm16	GACATTCCAATCCCACCAGA	CACCTCTGTATCCGTCAGCA
Retnla	TCAGCAATCCCATGGCGTATAA	TCATCAGTATTCACTGGGACCATCA
Tgfb1	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
Tgfb2	TCGACATGGATCAGTTTATGCG	CCCTGGTACTGTTGTAGATGGA
Tgfb3	CCTGGCCCTGCTGAACTTG	TTGATGTGGCCGAAGTCCAAC
Tgfbr1	TGTGCACCATCTTCAAAAACA	ACCAAGGCCAGCTGACTG
Tnfa	GTAGCCCACGTCGTAGCAAAC	CTGGCACCACTAGTTGGTTGTC
Ucp1	TACCAAGCTGTGCGATGTCCA	GCACACAAACATGATGACGTTCC
36b4	GAGGAATCAGATGAGGATATGGGA	GAGGAATCAGATGAGGATATGGGA

and 0.5 mM isobutyl methylxanthine) for 2 days and subsequently in DMEM-based growth medium containing 20 nM insulin and 1 nM triiodothyronine for an additional 7 days in either the presence or absence of GDF1. Cells were stained with Oil Red O using standard procedures as described elsewhere [13].

The murine pre-adipocytic cell line 3T3-L1 cells were cultured with 20 nM insulin, 0.5 μM dexamethasone and 0.5 mM isobutyl methylxanthine for 2 days and subsequently were cultured with 20 nM insulin alone for an additional 2 days. The murine myoblastic cell line C2C12 cells were cultured in DMEM containing 10% FBS, and were used without myogenic differentiation.

To stimulate hypoxia, cells were cultured in dishes placed in a jar containing an AnaeroPack™-Anaero Anaerobic Gas Generator (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37 °C for 12 h, depending on the experimental protocol, according to the manufacturer's instructions. The oxygen concentration reached a level of less than 1% within 1 h [14].

Cultured cells were incubated with 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). Afterward, 0.04 M HCl in isopropyl alcohol was added to the cultured cells to dissolve the formazan. The dissolved suspension was assayed using an enzyme-linked immunosorbent assay and the absorbance at a wavelength of 550 nm was measured using a microplate reader.

2.4. Culture of macrophages and migration assay

RAW264.7 cells were cultured in DMEM supplemented with FBS as previously described [15]. For preparation of peritoneal macrophages, 3 mL of sterile phosphate-buffered saline (PBS) was injected into the peritoneal cavity of mice and then lavage fluids were collected and centrifuged at 200*g* for 5 min. The resultant pellet was subsequently washed with PBS. The obtained macrophages were again centrifuged, suspended in DMEM, seeded on dishes, and allowed to adhere for 2 h. Floating cells were removed by washing and adherent cells were used as peritoneal macrophages in the experiments.

RAW264.7 cells or peritoneal macrophages were seeded on 8 μm-pore chemotaxis membranes. Medium containing 100 ng/mL of GDF-1 was added to the basolateral side of the membrane and the cells were allowed to migrate for 16 h. At the end of the assay, the filter was removed, fixed in methanol, and stained with Giemsa stain. The filters were then placed on glass slides and the cell pellets corresponding to the upper wells were wiped off with cotton swabs. The number of cells that migrated to the basolateral side of the chamber was counted.

2.5. Statistical analysis

Results are all expressed as the mean ± standard error of the mean (SEM) and the statistical significance was determined by the two-tailed and unpaired Students' *t*-test or the one-way analysis of variance (ANOVA) with the Holm/Sidak post-hoc test.

3. Results

3.1. GDF1 is preferentially downregulated in BAT of obese mice

In accordance with the upregulation of *GDF5* expression [12], we comprehensively searched for responsive BMP/GDF family member genes in the BAT of leptin-deficient, 12-week-old, *ob*/*ob* mice as a model of genetic obesity (WT mice 24.2 ± 0.3 g; *ob*/*ob* mice 53.1 ± 0.8 g). In addition to GDF5, significant upregulation was observed in the expression levels of transforming growth factor β1 (*Tgfb1*), *BMP3*, and inhibin β_B (*Inhbb*) in interscapular BAT of *ob*/*ob* mice, whereas among the different BMP/GDF family members, a significant decrease was only observed in *GDF1* expression in BAT of *ob*/*ob* mice (Fig. 1A). However, no significant change was found in the expression levels of the other tested member genes. Although constitutive *GDF1* expression was detected in bone, muscle, and WAT, but not in the liver or pancreas, *GDF1* expression was not significantly altered in these tissues, but constitutively expressed in *ob*/*ob* mice (Fig. 1B). ELISA results showed that GDF1 levels in BAT of *ob*/*ob* mice were significantly decreased (Fig. 1C). We next examined whether acquired obesity similarly deteriorates *GDF1* expression in BAT as observed in genetically obese mice. Briefly, 8-week-old WT mice were maintained with either normal chow (NC) or a HFD for 1 and 2 consecutive months as a model of DIO. Body weight was significantly higher in mice fed a HFD than in that of mice fed a NC (Fig. 1D). The expression of specific marker genes of both M1 (monocyte chemoattractant protein-1 (*Mcp1*) and tumor necrosis factor-α (*Tnfa*)) and M2 (arginase 1 (*Arg1*) and resistin-like molecule-α (*Retnla*)) macrophages was markedly increased in BAT of mice fed a HFD for 2 months (Fig. 1E), while the expression of *Ucp1* was significantly increased in BAT of mice fed a HFD for 2 months (Fig. 1F). As observed in genetically obese *ob*/*ob* mice, *GDF1* expression was significantly downregulated in BAT of mice fed with HFD, as compared to mice fed with NC (Fig. 1G). However, the expression of *GDF1* (Fig. 1H) and macrophage markers (Supplemental Fig. 1) in BAT of mice fed a HFD was not altered when the mice were maintained under cold condition for 6 h. Moreover, GDF1 protein levels were significantly lower in BAT of mice fed a HFD as seen in *ob*/*ob* mice (Fig. 1I), along with a trend toward the lower levels of plasma GDF1 without statistical significance (Fig. 1J).

Next, we investigated whether obesity affects the expression of BMP/GDF receptors in BAT. In BAT from *ob*/*ob* mice, a significant decrease was only seen in the expression of *activin A receptor, type 1C* (*Acvr1c*) amongst different relevant genes examined, while HFD did not induce any significant alterations of mRNA expression of BMP receptors (Supplemental Fig. 2). Accordingly, GDF1 would be only one endogenous factor commonly down-regulated in BAT within molecules responsible for the input of extracellular BMP/GDF signals in both genetic and acquired obese mice.

3.2. GDF1 expression is decreased by various stressors in brown adipocytes

Because obesity is highly associated with the pathophysiology of various types of cellular stresses, we next determined whether GDF1 expression was responsive to different stressors relevant to hypoxic, endoplasmic reticulum (ER), mitochondrial, and oxidative stresses in cultured brown adipocytes *in vitro*. For this purpose, we first determined cellular viability using the MTT assay in brown adipocytes cultured with various stressors at different concentrations for 12 h and found significant inhibition of MTT reducing activity in brown adipocytes exposed to the ER stressor tunicamycin at 0.1–1.0 μg/mL (Fig. 2A), the mitochondrial stressor 2,4-dintriohenol at 1.0 mg/mL (Fig. 2B), and the oxidative stressor hydrogen peroxide at 500 μM (Fig. 2C), respectively. However, MTT reduction was not altered in cells cultured under hypoxic conditions for 12 h (Fig. 2D). These stressors could cause a plethora of cellular effect. Accordingly, brown adipocytes were then treated with tunicamycin at 0.01 μg/mL, 2,4-dintriohenol at 0.1 mg/mL and hydrogen peroxide at 100 μM, to observe whether these stressors could alter *GDF1* expression without affecting cell viability. A significant downregulation of *GDF1* expression was observed in brown adipocytes exposed to 0.01 μg/mL of tunicamycin (Fig. 2E) and 100 μM hydrogen peroxide (Fig. 2G), while neither 2,4-dintriohenol (Fig. 2F) nor hypoxia (Fig. 2H) significantly

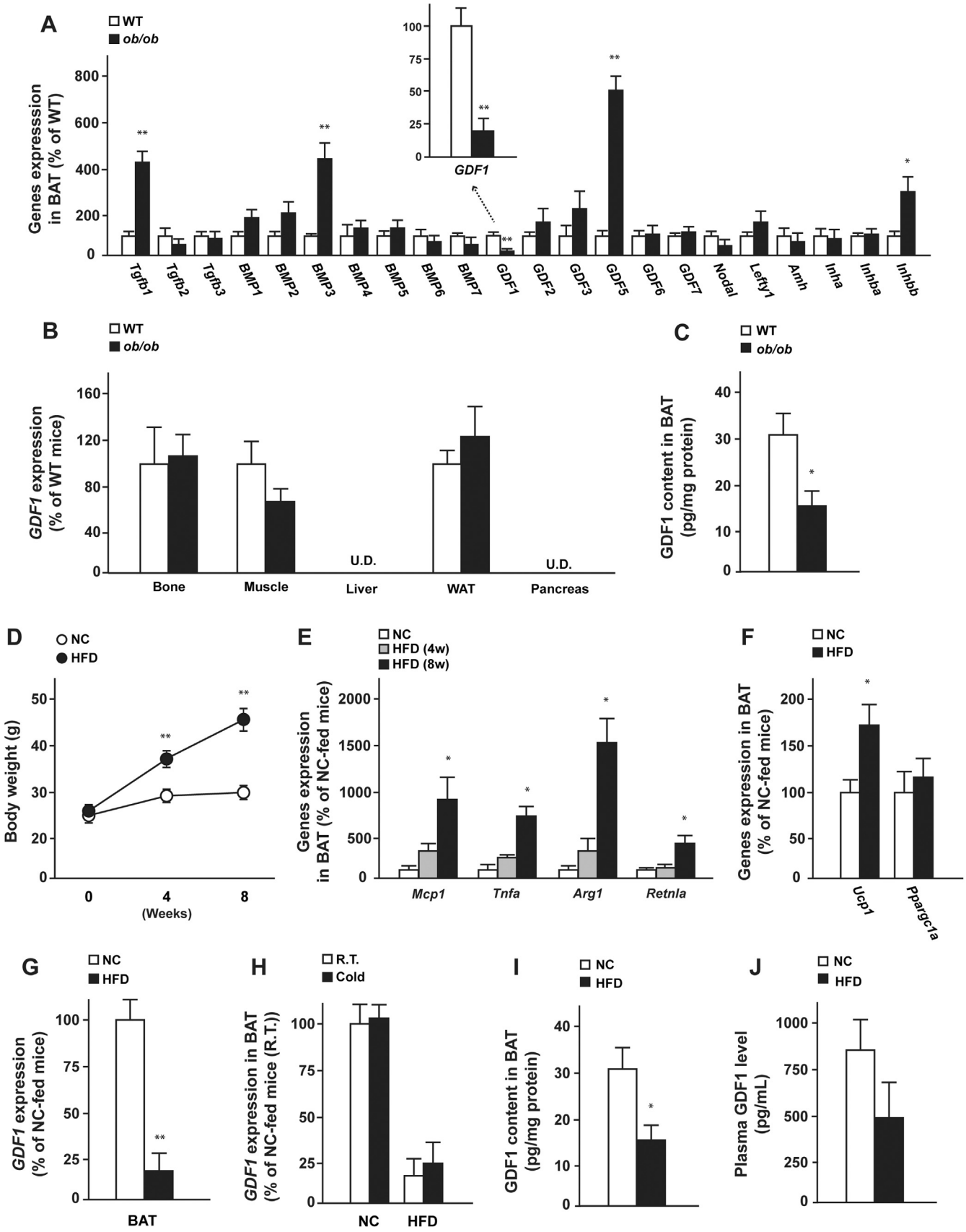

Fig. 1. GDF1 is preferentially down-regulated in BAT of obesity model mice. Tissues were isolated from *ob/ob* mice at 12 weeks of age, followed by determination of (A, B) mRNA expression by qPCR and (C) GDF1 levels by ELISA ($n=4$–6). $^{*}P<0.05$, $^{**}P<0.01$, significantly different from each control value obtained in WT mice. Adult male WT mice at 8 weeks old were fed with either NC or HFD for 2 months, followed by determination of (D) body weight and (E–G) mRNA expression in BAT by qPCR ($n=4$). (H) Adult male mice fed a NC or HFD were maintained in cold condition for 6 h, and subsequent determination of mRNA ($n=4$). GDF1 levels in (I) BAT and (J) plasma of HFD-fed mice ($n=4$–8). $^{*}P<0.05$, $^{**}P<0.01$, significantly different from the value obtained in NC-fed mice.

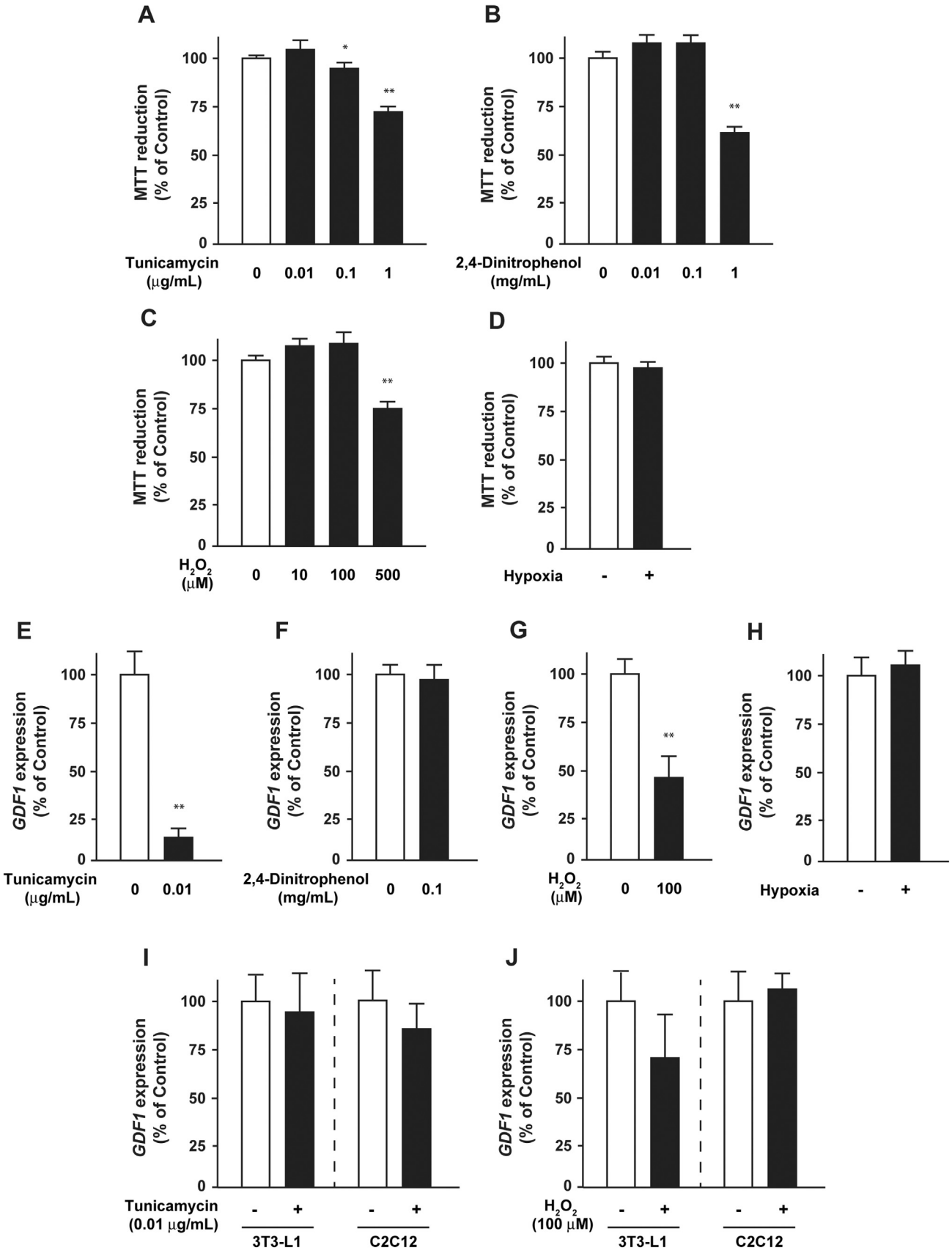

Fig. 2. *GDF1* expression is down-regulated by ER stress and oxidative stress in brown adipocytes. Brown adipocytes were cultured for 12 h with (A) tunicamycin at 0.01–1 μg/mL, (B) 2,4-dinitrophenol at 0.01–1 mg/mL and (C) hydrogen peroxide at 10–500 μM, followed by determination of cell viability with MTT assay ($n=4$). $^{*}P<0.05$, $^{**}P<0.01$, significantly different from each control value obtained in cells cultured without stressors. Brown adipocytes were cultured for 12 h with (E) tunicamycin at 0.01 μg/mL, (F) 2,4-dinitrophenol at 0.1 mg/mL and (G) hydrogen peroxide at 100 μM, followed by determination of *GDF1* expression with qPCR ($n=4$–6). (H) Brown adipocytes were cultured under hypoxia for 12 h, followed by determination of *GDF1* expression ($n=4$). $^{**}P<0.01$, significantly different from each control value obtained in cells cultured without stressors. White adipocytic 3T3-L1 cells or myoblastic C2C12 cells were similarly cultured for 12 h with (I) 0.01 μg/mL tunicamycin or (J) 100 μM hydrogen peroxide, followed by determination of *GDF1* expression with qPCR ($n=4$).

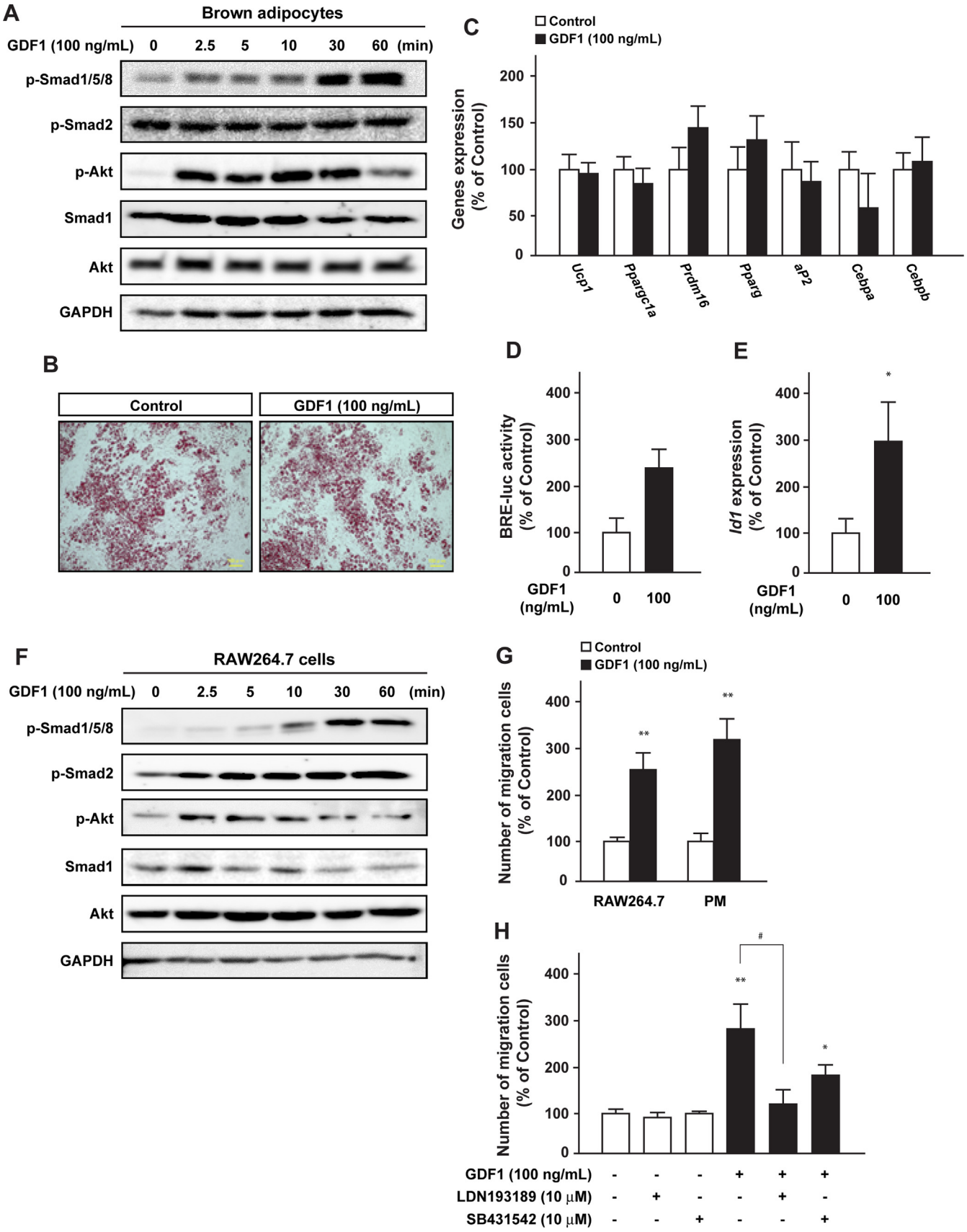

Fig. 3. GDF1 does not affect brown adipogenesis but stimulates macrophage migration through BMP receptor**:** (A) brown adipocytes were exposed to 100 ng/mL GDF1 for different periods indicated, followed by determination of the phosphorylation of downstream mediators such as Smad and Akt. Representative images are shown. Brown adipocytes were cultured with recombinant GDF1 at 100 ng/mL for 7 days, followed by determination of (B) Oil red O staining, (C and E) mRNA expression by qPCR, and (D) BRE-luc activity ($n=4$). (F) Macrophagic RAW264.7 cells were exposed to 100 ng/mL GDF1 for different periods indicated, followed by determination of the phosphorylation of downstream mediators such as Smad and Akt. Representative images are shown. (G) RAW264.7 cells and peritoneal macrophages (PM) were seeded onto chemotaxis membranes, followed by addition of medium containing 100 ng/mL GDF1 to the basolateral side of the membrane, and subsequent culture for 16 h for determination of the number of macrophages migrated ($n=6$). (H) RAW264.7 cells were pre-treated with LDN193189 or SB431542 at 10 μM, followed by culture with 100 ng/mL GDF1 for 16 h and subsequent determination of the number of cells migrated ($n=3$–6). $^{*}P<0.05$, $^{**}P<0.01$, significantly different from the value obtained from cells not treated with GDF1. $^{\#}P<0.05$, significantly different from the value obtained from cells treated with GDF1 alone.

affected *GDF1* expression in brown adipocytes under these experimental conditions. In contrast to brown adipocytes, neither tunicamycin (Fig. 2I) nor hydrogen peroxide (Fig. 2J) significantly alter *GDF1* expression in either white adipocytic 3T3-L1 cells or myoblastic C2C12 cells.

3.3. GDF1 did not affect differentiation of brown adipocytes, but stimulated migration of macrophages

We next investigate whether GDF1 modulates differentiation of brown adipocytes as observed with GDF5 [16]. Although exposure to GDF1 facilitated phosphorylation of Smad1/5/8 and Akt (Fig. 3A), as observed in GDF5-treated brown adipocytes [12], GDF1 failed to significantly affect accumulation of lipid droplets stained with Oil Red-O (Fig. 3B) or expression profiles of several marker genes, including *Ucp1*, PPARγ co-activator 1α (*Ppargc1a*), PRD1-BF-RIZ1 homologous domain containing protein-16 (*Prdm16*), *Pparg*, adipocyte protein 2 (*aP2*), CCAAT enhancer-binding protein α (*Cebpa*), and *Cebpb*, in cultured brown adipocytes (Fig. 3C). On the contrary, GDF1 significantly enhanced the reporter activity of BRE-luc, a luciferase plasmid with BMP responsive element (Fig. 3D) and increased the expression of inhibitor of differentiation 1 (*Id1*), a major downstream effector of BMP signaling (Fig. 3E), indicating that GDF1 indeed could turn on BMP/Smad signal transduction in brown adipocytes.

Macrophage infiltration into adipose tissues has been well documented in obese mice and humans [17,18], whereas particular BMP/GDF family members were shown to be responsible for migration of macrophages rather than modulation of adipocyte differentiation [19]. Thus, we next investigated whether GDF1 affects migration of macrophages *in vitro* and found that exposure to GDF1 led to marked phosphorylation of both Smad1/5/8 and Akt in macrophagic RAW264.7 cells (Fig. 3F), similar to that in brown adipocytes (Fig. 3A). A significant increase in the number of migrated cells was observed with RAW264.7 cells and peritoneal macrophages after culturing with GDF1 (Fig. 3G). LDN193189, an inhibitor of activin-like kinase 2/3 (ALK2/3), almost completely inhibited the migration of cells increased by GDF1, whereas SB431542, an inhibitor of ALK4/5/7, failed to significantly inhibit the GDF1-induced increase in the number of migrating cells at the concentration used (Fig. 3H). The treatment of LDN193189 or SB431542 alone did affect the number of migrating cells (Fig. 3H). These results indicate that GDF1 promotes macrophage migration through the activation of the receptor complex between BMP type-I receptor composed of ALK2 or ALK3 and BMP type-II receptor in macrophages.

4. Discussion

In this study, we identified GDF1 as an endogenous factor downregulated in BAT of both genetic and acquired obesity mice. The current findings that GDF1 promoted macrophage migration without affecting brown adipogenesis *in vitro*, and GDF1 protein level was significantly decreased in BAT but not in circulation of obese mice *in vivo*, gave rise to the notion that GDF1 is usually secreted from brown adipocytes to promote macrophage infiltration into BAT *in vivo* as a paracrine factor instead of endocrine factor. Taking into consideration the prevailing view that obesity induces marked macrophage infiltration into adipose tissues [17,18], it is conceivable that downregulation of GDF1 expression could lead to a feedback compensation to counteract extraordinarily excessive infiltration of adjacent macrophages into BAT in an obesogenic situation, as shown in Supplemental Fig. 3. Future analyses using conditional knockout mice defective of GDF1 from BAT will undoubtedly help to further elucidate the physiological and pathological roles of this particular BMP/GDF family member expressed by macrophage infiltrating brown adipocytes during obesity *in vivo*. The differential sensitivities to ALK inhibitors are suggestive of an idea that extracellular GDF1 promotes macrophage migration through activation of the receptor complex between BMP type-I receptor composed of ALK2 or ALK3 and BMP type-II receptor in macrophages.

It should be emphasized that GDF1 failed to affect cell vitality and differentiation in brown adipocytes in spite of increased Smad1/5/8 phosphorylation, which is required for promotion by GDF5 of brown adipogenesis [20]. In addition to activation of the Smad pathway, BMP/GDF family members are shown to utilize various intracellular signaling pathways to transduce their signals essential for regulation of a variety of biological activities [21,22]. BMP7 accelerated a full program of brown adipogenesis through activation of p38 mitogen-activated protein kinase (MAPK) [10], for example, while BMP8B centrally or peripherally enhanced BAT thermogenesis along with activation of either AMP-activated protein kinase (AMPK) or p38 MAPK [11]. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is also involved in GDF5-induced brown adipogenesis [16]. Accordingly, BMP/GDF family would sophisticatedly regulate brown adipogenesis through activation of various intracellular signaling pathways including Smad1/5/8, PI3K/Akt, p38 MAPK and AMPK. Although the exact reason why GDF1 failed to promote brown adipogenesis despite activation of the Smad pathway is not clarified so far, the paradoxical property between GDF1 and GDF5 on brown adipogenesis could be at least in part accounted for by taking into consideration the complex signal crosstalk among a variety of intracellular pathways in brown adipocytes.

The reason why GDF1 expression was downregulated in brown adipocytes exposed to ER stress and hydrogen peroxide *in vitro*, as well as in BAT of inborn and acquired obesity mice *in vivo*, has not yet been clarified. The Smad2/3 pathway reportedly plays a role in mechanisms underlying TGF-β1-mediated downregulation of GDF1 expression in peripheral blood mononuclear cells [23]. The ER stressor tunicamycin induced Smad2/3 phosphorylation in alveolar epithelial cells [24], whereas oxidative stress indirectly promoted Smad2 phosphorylation in myofibroblasts [25]. These previous findings indicate that activation of the Smad2/3 signaling pathway would be at least in part responsible for the predominant downregulation of GDF1 expression in brown adipocytes in response to obesogenic signals amongst a variety of BMP/GDF family members in a particular situation. *In silico* analysis of the upstream promoter regions of *GDF1* along with reporter plasmid assays will be undoubtedly beneficial for further clarification in future studies. It is conceivable that a repressor type of a particular nuclear transcription factor would be upregulated to participate in the predominant downregulation of GDF1 in brown adipocytes under obesity.

Although evidence is accumulating for macrophage infiltration into WAT in obesity and relevant diabetes mellitus, little attention has been paid to the possible importance of macrophages in the physiology and pathophysiology of BAT during obesity. However, macrophages have been recently shown to be essential for heat production and adaptive thermogenesis by producing noradrenaline in BAT [26]. Moreover, macrophages may contribute to the development of functional beige cells in subcutaneous WAT [27]. The present findings that genetic and acquired obesity similarly downregulated expression of GDF1, which is capable of accelerating macrophage migration in BAT rather than WAT, led us to speculate that the number of infiltrating macrophages would be reduced in BAT through a mechanism associated with GDF1 downregulation toward decreased energy expenditure by brown adipocytes during obesity. Future analysis of macrophage infiltration into BAT of obese animals *in vivo*, as well as identification of

the type of macrophages infiltrated, will undoubtedly aid in determining a solution to this concern. Pharmacological manipulations of the GDF1/BMP receptor pathway in macrophages as well as GDF1 secretion from brown adipocytes presents a plausible approach for the discovery and development of novel strategies to combat obesity and related metabolic diseases in human beings.

Conflict of interest

The authors have declared that no competing interests exist.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.12.008.

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