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Response of human mature adipocytes to hypoxia-reoxygenation

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Abstract

Background aims. Adipocytes are metabolically active cells and have endocrine functions, such as cytokine secretion. Notably, adipocytes are found underneath skin and are thought to be involved in the body's response to ischemia-reperfusion (I-R). I-R injury is an important factor in the pathogenesis of chronic skin wounds. In this study, we investigated the response of human adipocytes to hypoxia-reoxygenation (H-R), the *in vitro* equivalent of I-R. *Methods.* We cultured human mature adipocytes by enclosing them in hydrogel composed of hyaluronan and collagen and analyzed their proliferation and response to H-R. *Results.* The average diameter of mature adipocytes isolated from abdominal subcutaneous fat tissue was between 60 and 109 μm, and a positive correlation was found between adipocyte size and body mass index. Hydrogel-enclosed human adipocytes displayed viability in *in vitro* culture and were capable of expressing foreign genes for at least 1 month. Proliferation analysis revealed 5-bromo-2′-deoxy-uridine labeling and positive Ki67 signaling. vascular endothelial growth factor expression was differentially altered in adipocytes in response to hypoxia and H-R. Adipocyte messenger RNA expression of pro-inflammatory cytokines, such as interleukin-1, interleukin-8 and tumor necrosis factor-α, was upregulated in response to H-R. In addition, the expression of heat shock protein 70, a cytoprotective gene, and inducible nitric oxide synthase, a proapoptotic gene, were both increased in H-R. Survival of hydrogel-enclosed adipocytes was found at 2 months after delivery into athymic mice. *Conclusions.* These and previous results from our group show that mature adipocytes can be cultured *in vitro* within a matrix and that they are functionally active cells that respond to environmental changes.

Key Words: *cytokine, hydrogel, hypoxia-reoxygenation, mature adipocytes, proliferation*

Introduction

Adipose tissues are primarily found underneath skin (subcutaneous fat) and around internal organs (visceral fat). They are primarily composed of adipocytes and exist in two forms, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT stores energy in the form of triglycerides and BAT dissipates stored energy by producing heat [1,2]. Recent studies have revealed endocrine functions for adipocytes, including the secretion of adipokines, which are involved in autocrine and paracrine signaling [3–6]. Dysregulation of adipokine expression or secretion can result in inflammation and obesity-related metabolic disorders, such as hypertension, stroke, cardiovascular disease and type 2 diabetes [7–9]. Adipose tissues also contain other cell types, such as monocytes/macrophages, endothelial cells and pre-adipocytes. Adipocytes in adipose tissue are surrounded by an extracellular matrix composed of collagen, hyaluronic acid and elastin [10–12].

Cytokines secreted by mature adipocytes are called adipokines and include tumor necrosis factor-α (TNF-α) and interleukin (IL)-6. Adipocytes also secrete adipose-specific factors, such as leptin, adiponectin and resistin [2–4,6,13,14]. Adipokines relay information to different organs, including the liver, brain and β-islet cells. Dysregulation of adipokines results in a chronic low-grade inflammation, which could lead to atherosclerosis, hypertension and type 2 diabetes [3,6,15,16]. Environmental signals such as nutrient intake affect the volume of adipose tissues, which suggests that adipose tissues are capable of plasticity [17–19]. An understanding of the mechanism behind adipocyte proliferation and differentiation is essential for the study of obesity and diabetes. Traditionally, mature adipocytes have been studied

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by differentiating primary pre-adipose cells or established pre-adipocyte cell lines that readily attach to the plastic plates used in cell culture. The ability to culture mature adipocytes harvested from tissue has been limited by the buoyancy of these cells. Differentiation of primary pre-adipose cells and pre-adipocyte cell lines is achieved with the use of an adipogenic cocktail containing isobutylmethylxanthine, dexamethasone and insulin [20,21]. However, differentiated adipocytes are different from mature adipocytes in that they contain multilocular lipid droplets. In contrast, mature adipocytes in WAT have a unique structure in which one large lipid droplet occupies the entire cytoplasm and pushes the nucleus peripherally. Recently, we established a novel *in vitro* method to culture mouse mature adipocytes by enclosing them in a hydrogel [22].

Ischemia-reperfusion (I-R) has been studied extensively in the brain, heart, liver and kidney. I-R injury is also an important factor in the pathogenesis of chronic skin wounds [23,24]. Ischemia leads to tissue compromise and eventually, death. Restoration of blood flow (tissue reperfusion) is necessary to prevent continued tissue injury and death. Paradoxically, reperfusion can lead to additional tissue compromise. One of the major molecular mechanisms thought to mediate I-R injury is the generation of oxidative stress on reperfusion. In I-R states, highly reactive oxygen species inflict damage on cellular structures. This direct cellular damage is further augmented by injury from the ensuing inflammatory cascade. In diabetic ulcers and pressure sores, direct pressure over a localized area is followed by reperfusion, and this cycle is often repeated. In venous ulcers, the arteriovenous gradient in a leg is lost when the leg is dependent, resulting in blood stasis and loss of perfusion. Perfusion is restored when the leg is elevated and venous hypertension is relieved [24]. Reperfused tissues are subjected to oxidative damage, which may contribute to the development of chronic wounds [25,26]. Recent data from our group with the use of a model of subclinical, cyclic I-R injury in rabbit ears showed a consistent, quantitative impairment in wound healing and altered gene expression [27].

We recently showed that mouse mature adipocytes play a critical role in stress response [22]. Expression of hypoxia-regulated genes, including vascular endothelial growth factor (VEGF) and heme oxygenase 1 was upregulated in response to hypoxia in mouse adipocytes. Here we report that human adipocytes can be cultured by enclosing them in hydrogel. We show that human adipocytes respond to hypoxia-reoxygenation (H-R) *in vitro*, which is the equivalent of I-R injury *in vivo*. Our current and previous reports suggest that mature adipocytes are functionally active cells that respond to environmental changes.

Methods

Isolation of human mature adipocytes

Human subcutaneous fat tissues collected from elective abdominoplasties were used to isolate mature adipocytes. Human tissues were obtained according to the guidelines of a protocol approved by the Institutional Review Board of Northwestern University. Mature adipocytes were isolated as previously described with some modifications [22]. Briefly, 5 mL of the fat tissues were minced and were then treated with collagenase II (0.25%, dissolved in Hank's buffered salt solution [HBSS], Gibco-BRL, Grand Island, NY, USA) in a final 10 mL for 1 h at 37°C in a shaking water bath. After complete digestion, 5 mL of HBSS was added to the reaction and mixed by inverting. The stromal vascular fraction, which contains mesenchymal cells (pre-adipocytes), fibroblasts and erythrocytes, was separated from mature adipocytes by taking advantage of the buoyant properties of adipose cells. The mixture was centrifuged for 10 min at 300*g* at room temperature, and approximately 2 mL of the floating layer containing mature adipocytes was recovered and resuspended in 13 mL of new HBSS. This step was repeated for an additional 2 cycles to ensure complete removal of the stromal vascular fraction from the mature adipocytes. The diameter of mature adipocytes was determined by analyzing the captured image of mature adipocytes by means of light microscopy, with the use of the NIH ImageJ program as previously described [22].

In vitro culture of mature adipocytes

Mature adipocytes were enclosed in a hydrogel matrix (Extracel-HP, BioTime Inc, Alameda, CA, USA) and cultured *in vitro* as previously described [22]. We used a ratio of 2:2:1 for Glycosil:Gelin:Extralink with 10% human mature adipocytes. Dulbecco's modified Eagle's medium/F12 medium containing 10% fetal bovine serum was used as a culture medium, which was changed every 2–3 days. Hypoxia (1% O_2) was achieved with the use of a hypoxic chamber (Invivo2 1000, Ruskinn Technology, Sanford, ME, USA).

Viability test and green fluorescence protein expression in mature adipocytes

Viability of isolated mature adipocytes was assessed by means of 0.4% trypan blue vital staining [22,28]. The LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, Carlsbad, CA, USA) was used to determine the viability of mature adipocytes. Samples were treated with red fluorescent ethidium homodimer-1 (2.5 μmol/L) and green fluorescent calcein-AM (1 μmol/L)

for 20 min at room temperature. Signal was detected with the use of a fluorescent microscope.

Adipocytes were transduced with a lentivirus (LV-GFP) in which GFP expression is driven by a cytomegalovirus promoter. Equal volumes of lentivirus (7×10^7 infectious units/mL, 10 µL) and adipocytes (10 µL) were mixed in the presence of 6 µg/mL of polybrene and enclosed in hydrogel. The mixture was cultured as described, and GFP expression was analyzed under a fluorescent microscope.

Reverse transcription—quantitative polymerase chain reaction and Western blot analysis

Adipocytes in hydrogel were homogenized for 90 seconds at 5000 rpm in a MagNA Lyser homogenizer (Roche, Indianapolis, IN, USA) with the use of the Zirconia beads (2.0-mm diameter, Biospec Products Inc, Bartlesville, OK, USA) in the presence of Tri Reagent (Sigma-Aldrich, St Louis, MO, USA). After genomic DNA was removed with the use of the Turbo DNA-free kit (Ambion, Austin, TX, USA), complementary DNA was made from total RNA with the use of Superscript II (Invitrogen, Carlsbad, CA, USA) with random primers. Polymerase chain reaction (PCR) was performed to detect expression of messenger RNA (mRNA). For the quantitative analysis, quantitative PCR (qPCR) analyses with the use of SYBR green I were performed with the use of an ABI prism 7000 sequence detection system or 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Expression of each gene was normalized to the level of β-actin to get a ΔCt. The $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression difference between differentiated and control samples. Primer sets are shown in Table I.

For Western blot analysis, proteins were prepared with the use of Tri Reagent according to the manufacturer's protocol. Isolated proteins were resuspended in 1% sodium dodecyl sulfate, run on a sodium dodecyl sulfate polyacrylamide gel and transblotted on nitrocellulose membranes. Membranes were incubated with anti-VEGF antibody (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then were incubated with horseradish peroxide—conjugated secondary antibody (1:5000 dilution; Vector Laboratories, Burlingame, CA, USA). Specific bands were visualized through the use of an enhanced chemiluminescence detection kit (GE Healthcare, Piscataway, NJ, USA). The blots were probed with anti—β-actin antibody (1:5000 dilution; Sigma-Aldrich, St Louis, MO, USA) to serve as a control for gel loading.

5-Bromo-2′-deoxy-uridine and Ki67 staining

The adipocyte and hydrogel mixture was treated with 5-bromo-2′-deoxy-uridine (BrdU) labeling reagent (1:100 dilution, Invitrogen, Carlsbad, CA, USA) and cultured for 3 days. The matrix-adipocyte mixture was fixed overnight in 4% paraformaldehyde in phosphate-buffered saline at 4°C. The samples were treated with 30% sucrose overnight at 4°C before embedding in OCT compound and cut into 10-µm-thick sections. Incorporated BrdU signal was detected with the use of the BrdU staining kit (Zymed Laboratories, San Francisco, CA, USA) and visualized with the use of 3,3′-diaminobenzidine. Hematoxylin was used to visualize the hydrogel matrix. For Ki67 detection, mouse anti-Ki67 (1: 1,000 dilution, BD Biosciences, San Jose, CA, USA) was used as a primary antibody, and the signal was detected with the use of the Vectastain kit (Vector Laboratories,

Table I. Primer sequences used to detect human mRNA.

Gene	Sense	Antisense
IL-1β	CTCGCCAGTGAAATGATGGCT	TCGGAGATTCGTAGCTGGAT
MCP-1	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
TNF-α	ATGAGCACTGAAAGCATGATCC	GAGGGCTGATTAGAGAGAGGTC
IL-6	AAATTCGGTACATCCTCGACGG	GGAAGGTTCAGGTTGTTTTCTGC
IL-8	TTTTGCCAAGGAGTGCTAAAGA	AACCCTCTGCACCCAGTTTTC
IL-1α	ATCATGTAAGCTATGGCCCACT	CTTCCCGTTGGTTGCTACTAC
MIP	GTTCCTCTCCGAGCTCACC	TGCTGTAGGAGCGGTTCTG
Cxcl2	GCAGGGAATTCACCTCAAGA	GGATTTGCCATTTTTCAGCA
HO-1	GTCTTCGCCCCTGTCTACTTC	CTGGGCAATCTTTTTGAGCAC
iNOS	TCATCCGCTATGCTGGCTAC	CTCAGGGTCACGGCCATTG
Cox-2	GTGCAACACTTGAGTGGCTAT	AGCAATTTGCCTGGTGAATGAT
Hsp70	TTTGAGGGCATCGACTTCTACA	CCAGGACCAGGTCGTGAATC
Leptin	GAACCCTGTGCGGATTCTTGT	TCCATCTTGGATAAGGTCAGGAT
Adiponectin	GGCATGACCAGGAAACCAC	TTCACCGATGTCTCCCTTAGG
Fabp4	AGCACCATAACCTTAGATGGGG	CGTGGAAGTGACGCCTTTCA
β-Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

Burlingame, CA, USA) and visualized with the use of 3,3′-diaminobenzidine.

Delivery of hydrogel/human adipocytes to Nu/Nu mice

All animal experiments were performed under a protocol approved by the Northwestern University Institutional Animal Care and Use Committee. Isolated human mature adipocytes were enclosed in hydrogel (Hystem-C LS kit, BioTime Inc, Alameda, CA, USA) and delivered into immunodeficient mice (8–12-week-old male Nu/Nu mice, Charles River Laboratories, Wilmington, MA, USA). Four subcutaneous injections of 0.1 mL of mixture were delivered with the use of a 22-gauge needle (inner diameter, 0.413 mm). Skin samples injected with adipocyte/hydrogel mixture were harvested at 2 weeks and 2 months after injection. Six animals were analyzed for each time point. The mixture was fixed in formalin, embedded in paraffin blocks, and sectioned on a microtome at a thickness of 5 μm. The sections were stained with the use of hematoxylin and eosin.

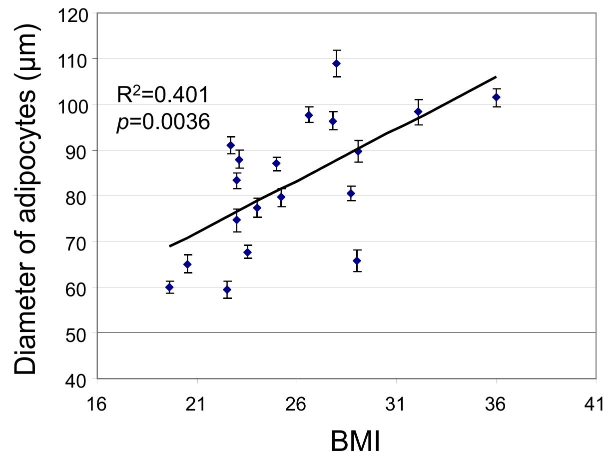

Figure 1. Correlation of BMI and adipocyte size. Mature adipocytes were isolated from human subcutaneous fat tissue and their size was measured with the use of the ImageJ program. Data are from 19 patients. More than 100 cells per sample were analyzed, and the diameter of adipocytes was presented as average ± standard error of the mean. Both BMI and diameter of adipocytes data were used to fit in a linear model performed by linear regression analysis in R. R^2 value of the model is 0.401; P value for F-test is 0.0036.

Results

Body mass index and the size of mature adipocytes show a positive correlation

We investigated the relationship between body mass index (BMI) and adipocyte size. Mature adipocytes were isolated from fat tissues obtained from female patients (BMI, 19.6–36.0; age 24–57 years). Because anatomic location can affect the size of mature adipocytes, we analyzed only abdominal subcutaneous fat tissues obtained from elective abdominoplasties. The average adipocyte diameter of the patients ranged from 60–109 μm (Figure 1). Statistical analysis showed a positive correlation between adipocyte size and BMI (Pearson correlation coefficient, 0.63; $R^2 = 0.40$; $P = 0.0036$).

Hydrogel-enclosed human mature adipocyte culture in vitro

We developed a novel culture method for mouse mature adipocytes, which otherwise cannot be cultured with the use of conventional methods because they float on the surface of culture media as the result of their high cytoplasmic lipid content [22]. We addressed whether human adipocytes can be cultured with the use of the method. First, we addressed the viability of mature adipocytes *in vitro*. Vital trypan blue staining showed that most mature adipocytes were viable after isolation (Figure 2A). In addition, live and dead staining with fluorescence dyes showed that most cells were viable after isolation (Figure 2C). Viability was found in most hydrogel-enclosed adipocytes after being cultured *in vitro* for 4 days (Figure 2D).

Next, we investigated whether foreign genes can be introduced to mature adipocytes. We were unable to determine the multiplicity of infection because isolated human mature adipocytes float in medium and are thus difficult to count. Instead, we mixed equal volumes of GFP-expressing lentiviruses with adipocytes and enclosed them in hydrogel. GFP signal was detected 3 days after culture (Figure 3D). The number of GFP-positive adipocytes increased continuously (day 16, Figure 3E). Although GFP-positive cells were detected (Figure 3F), the number of wrinkled adipocytes was increased at day 39 (Figure 3C). The number of GFP-expressing adipocytes was notably decreased at day 42 (data not shown).

Proliferation markers were found in human adipocytes cultured in vitro

It is known that mature adipocytes are terminally differentiated post-mitotic cells and that undifferentiated pre-adipocytes in adipose tissues proliferate in response to environmental stimuli such as food intake [29–32]. Recently, analysis of human adipose tissues has suggested that mature adipocytes proliferate [18,33]. We have previously shown that hydrogel-enclosed mouse mature adipocytes proliferate *in vitro* [22]. In the present study, we addressed whether human mature adipocytes proliferate. Hydrogel-enclosed adipocytes were cultured for 3 days in the presence of BrdU. Positive signals were found in

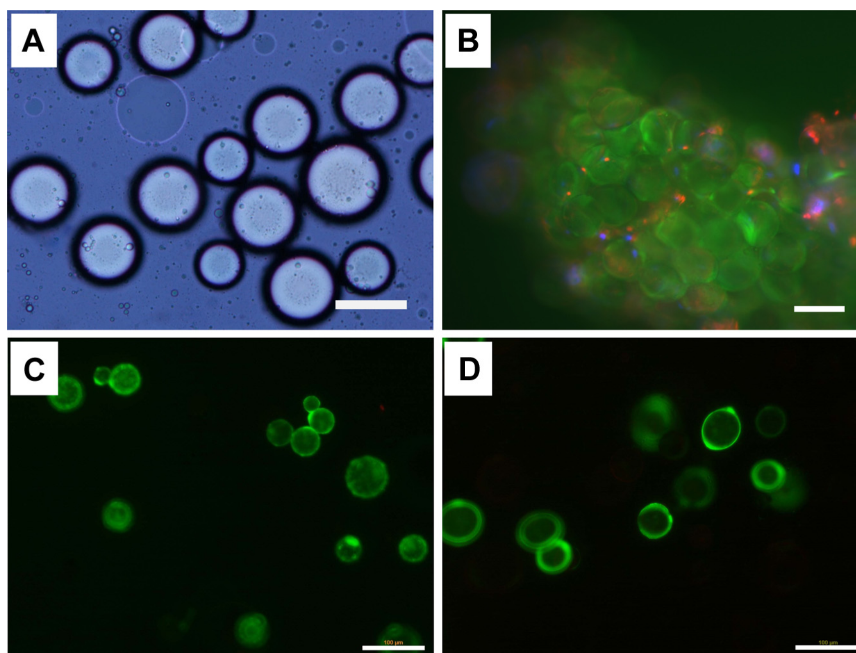

Figure 2. Viability of adipocytes. (A) Vital trypan blue staining. Isolated mature adipocytes after collagenase digestion were treated with 0.4% trypan blue. (B–D) LIVE/DEAD staining. Live and dead cells were stained by calcein-AM (green) and ethidium homodimer-1 (red), respectively. (B) Minced human fat tissue before collagenase digestion. Nuclei were stained with 4′-6-diamidino-2-phenylindole. (C) Adipocytes after isolation. (D) Hydrogel-enclosed adipocytes cultured for 4 days *in vitro*. Scale bar, 100 μm.

some adipocytes when fixed sections were analyzed with an anti-BrdU–specific antibody (Figure 4A). Analysis of another proliferation marker, Ki67, with an anti-Ki67 antibody showed proliferation of mature adipocytes in hydrogel (Figure 4B).

Expression of pro-inflammatory genes was upregulated in adipocytes in response to H-R

Previously we showed that mouse mature adipocytes are functionally active cells that respond to hypoxia *in vitro* [22]. We cultured hydrogel-enclosed human adipocytes in a hypoxic environment (1% oxygen) for 24 h. Expression of VEGF mRNA was increased by 3.2-fold after hypoxia treatment in human adipocytes compared with a normoxic control (Figure 5A). In line with mRNA expression, increased VEGF protein expression was found in human adipocytes after hypoxia treatment (Figure 5C, lane 1 versus lane 2). Next, we investigated the response of human adipocytes to H-R. VEGF expression in adipocytes was reduced by 50% in H-R at 3 h after reoxygenation (Figure 5B).

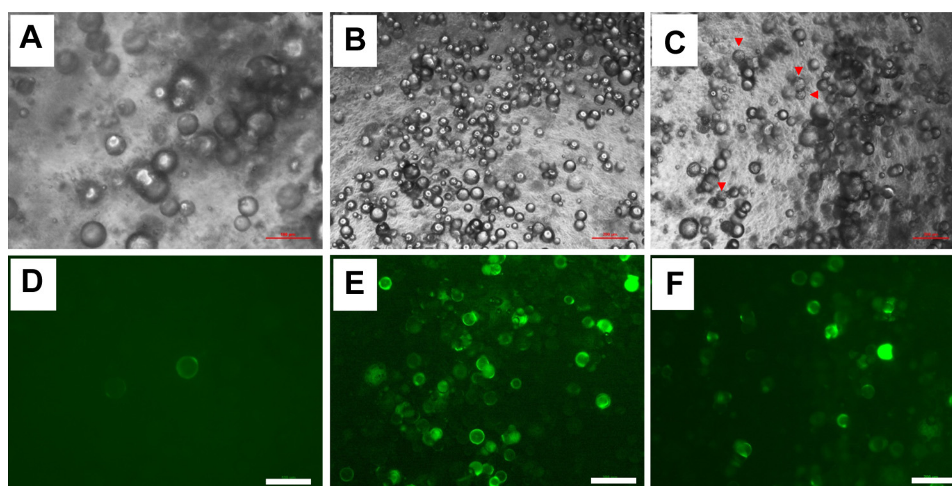

Figure 3. Delivery of a foreign gene in adipocytes. GFP-expressing lentiviruses were mixed with adipocytes, enclosed in hydrogel and cultured *in vitro*. (A–C) Bright field image. (D–F) Fluorescence image. (A, D) day 3, (B, E) day 16, (C, F) day 39 after culture. Wrinkled adipocytes are indicated by arrowheads. Scale bar, 100 μm (A, D), 200 μm (B, C, E and F).

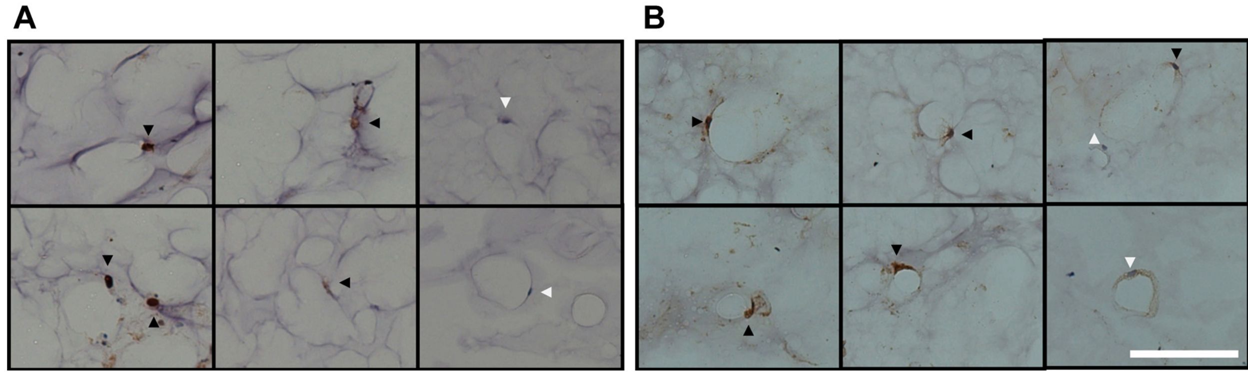

Figure 4. Human mature adipocytes proliferate *in vitro*. Adipocytes were enclosed in hydrogel, cultured *in vitro* in the presence of BrdU for 3 days and analyzed for the proliferation makers. (A) Anti-BrdU staining. (B) Anti-Ki67 staining. Hematoxylin was used to visualize the hydrogel after developing signals. Positive and negative signals in nuclei are indicated by closed and open arrowheads. Scale bar, 100 μm.

Decreased expression of VEGF mRNA and protein was found in adipocytes 24 h after reoxygenation when compared with hypoxia alone (Figure 5).

We analyzed the mRNA expression of various cytoprotective, pro-inflammatory and proapoptotic genes in adipocytes subjected to H-R (Figure 6). Whereas expression of heat shock protein (Hsp) 70, a cytoprotective stress response protein, was highly increased (8.9 ± 2.3−fold that of heme oxygenase 1), a heme catabolizing enzyme with cytoprotective properties was only marginally increased (1.3 ± 0.1−fold) by H-R. Expression of *iNOS*, a proapoptotic gene, and *Cox-2*, a pro-inflammatory gene, were upregulated by 7.0 ± 2.4−fold and 1.8 ± 0.3−fold, respectively, in response to H-R. Interestingly, the expression of many pro-inflammatory cytokines—IL-1β (3.0 ± 0.5−fold), monocyte chemoattractant protein 1 (MCP-1) (3.8 ± 0.9), TNF-α (5.1 ± 1.6), IL-6 (2.7 ± 0.5), IL-8 (5.6 ± 1.4), IL-1α (10.8 ± 3.5), macrophage inflammatory proteins (MIP) (1.8 ± 0.3)

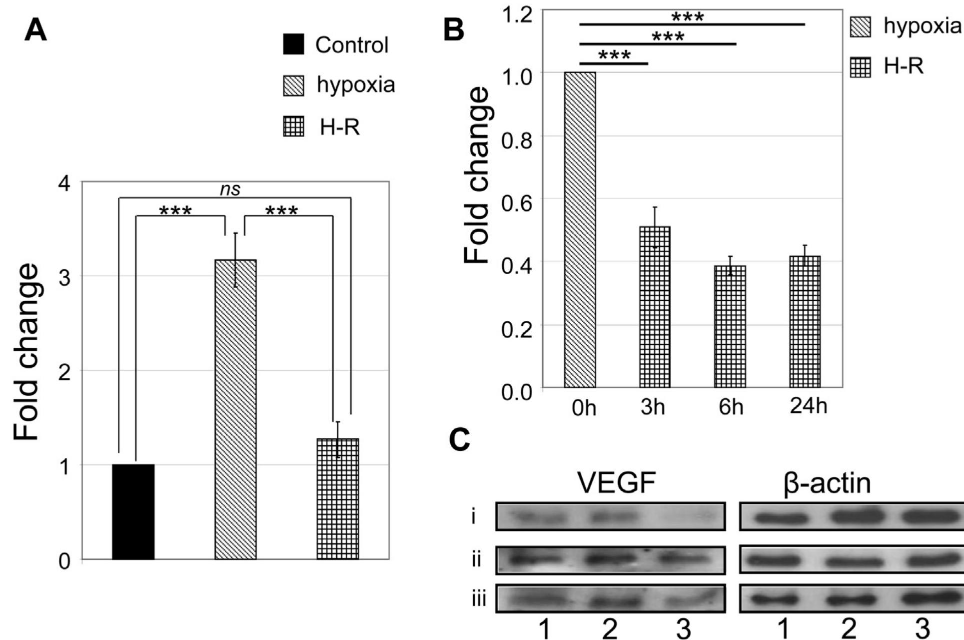

Figure 5. Human adipocyte response to H-R *in vitro*. Human mature adipocytes were cultured in hypoxic condition (1% oxygen) for 24 h. The culture was then placed in normoxic condition (21% oxygen, H-R) for 24 h (A, C) or as indicated (B). (A, B) VEGF mRNA expression. Total RNAs were isolated and reverse transcription (RT)-qPCR was performed. VEGF expression was normalized according to the expression level of β-actin. The level of VEGF expression in hypoxia and H-R was compared with that of normoxia, which was set at 1 (A). (B) The level of VEGF expression in H-R was compared with that in hypoxia, which was set at 1. Gene expression level was presented as a fold change. Data are from 19 patients (A) and 4−5 patients (B) and presented as average ± standard error of the mean. ***$P < 0.001$; ns, not significant. (C) Western blot analysis. Proteins were prepared human mature adipocytes; Western blot analyses were performed. Expression of VEGF was detected with its specific antibody. β-Actin was detected as a loading control. Western blots from 3 patients are shown (i, ii and iii). Lane 1, normoxia; lane 2, hypoxia; lane 3, H-R.

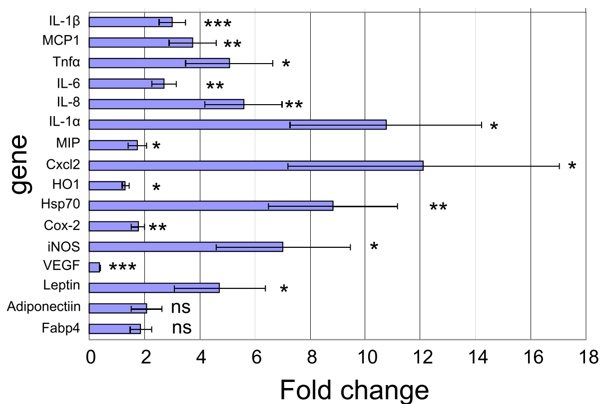

Figure 6. Messenger RNA expression of genes in adipocytes subjected to H-R. Hydrogel-enclosed human mature adipocytes were cultured in hypoxic condition (1% oxygen) for 24 h and placed in normoxic condition (21% oxygen, H-R) for 24 h. Total RNAs were isolated and RT-qPCR was performed. Individual gene expression was first normalized to corresponding levels of β-actin and was then presented as fold changes in H-R adipocytes relative to hypoxia adipocytes (average $\pm$ SEM). $n = 13-18$ (outliers, more than 2 standard deviations from the mean, were excluded for the analysis). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns, not significant.

and Cxcl2 (12.1 ± 4.9)— was also upregulated. We analyzed adipocyte-specific genes, including adipokines in H-R–treated adipocytes. Expression of leptin was upregulated by 4.8 ± 1.7–fold. Although the expression of adiponectin and Fabp4 was modestly increased, the change was not statistically significant.

Survival of hydrogel-enclosed human adipocytes in vivo

Fat autografts are commonly used to fill soft-tissue defects. However, long-term survival of transplanted fat is low, and the majority of grafted fat is absorbed by the body. Transplanted fat cells are also collapsed when delivered to non-native sites because a suitable microenvironment is not provided for their survival. Adipocytes *in vivo* exist in a 3-dimensional matrix that consists of hyaluronan and collagen. We investigated the behavior of adipocytes when adipocytes are delivered in conjunction with a 3-dimensional matrix *in vivo*. After optimizing the hydrogel-human adipocyte ratio, we found that Hystem-C LS with 20% adipocytes solidified in 20–25 min *in vitro*. The hydrogel-adipocyte mixture was placed into 1-mL syringes and continuously mixed in a rotary shaker until solidified to ensure a homogeneous distribution of mature adipocytes in hydrogel. We injected 0.1-mL aliquots of the mixture subcutaneously into 4 sites (shoulder and inguinal fat pad regions). We used athymic immunodeficient nude mice, which have been used for xenograft studies. The injected material was found remaining in the animals when analyzed 2 weeks and 2 months after injection (Figure 7A). The volume of mixture remaining was 36.1 μL at 2 weeks after injection (input was 99.2 μL). The volume was further reduced to 25.8 μL at 2 months after injection (Figure 7B). Similar results were found when measuring weight: 90.8, 32.4 and 20.5 mg for input, 2 weeks, 2 months samples, respectively (Figure 7B). Histological analysis showed that the adipocyte-hydrogel mixture was mostly intact when analyzed 2 weeks after injection (Figure 7C, left). Cells from the host were found in the adipocyte-hydrogel mixture, and host tissues were integrated into the mixture when analyzed 2 months after injection (Figure 7C, right).

Discussion

The buoyant nature of adipocytes has limited the culture of mature adipocytes *in vitro*. We have previously developed an *in vitro* method to culture mature mouse adipocytes with the use of hydrogel, a material composed of hyaluronan and collagen [22]. Hydrogel was used for two purposes; to provide a 3-dimensional matrix, which is the environment found in native adipose tissue, and to keep adipocytes from floating by encasing them. Adipocytes are metabolically active and function as endocrine cells by expressing and secreting many cytokine and hormones. We found that human adipocytes cultured *in vitro* respond to hypoxia and H-R (Figure 5). I-R (or H-R) injury generates reactive oxygen species, which causes cell damage and induces an inflammatory cascade [34,35]. H-R resulted in the upregulation of multiple pro-inflammatory cytokines in adipocytes including MCP-1, MIP, IL-1α, IL-1β, IL-6, IL-8, Cxcl2 and TNF-α (Figure 6). The expression of cytoprotective stress response protein, heat shock protein 70, was also increased. The expression of iNOS, which creates nitro oxide–free radicals, was also upregulated by H-R in adipocytes.

We investigated the possibility of performing gain-of-function studies in mature adipocytes. By enclosing lentiviruses and adipocytes in hydrogel, we were able to infect adipocytes with GFP-expressing lentivirus and achieve GFP expression in adipocytes (Figure 3). Although we were able to culture adipocytes *in vitro* with the use of hydrogel, we were unable to stock mature adipocytes in liquid nitrogen. This is one limitation to the use of mature adipocytes for adipogenesis studies compared with the use of primary pre-adipocytes or pre-adipocyte cell lines. It is thought that mature adipocytes are terminally differentiated cells that do not proliferate [31,32]. It has been suggested that a higher rate of proliferation in pre-adipocytes or increased adipocyte volume in response to environmental stimuli causes obesity. However, mature adipocytes have the potential to proliferate because

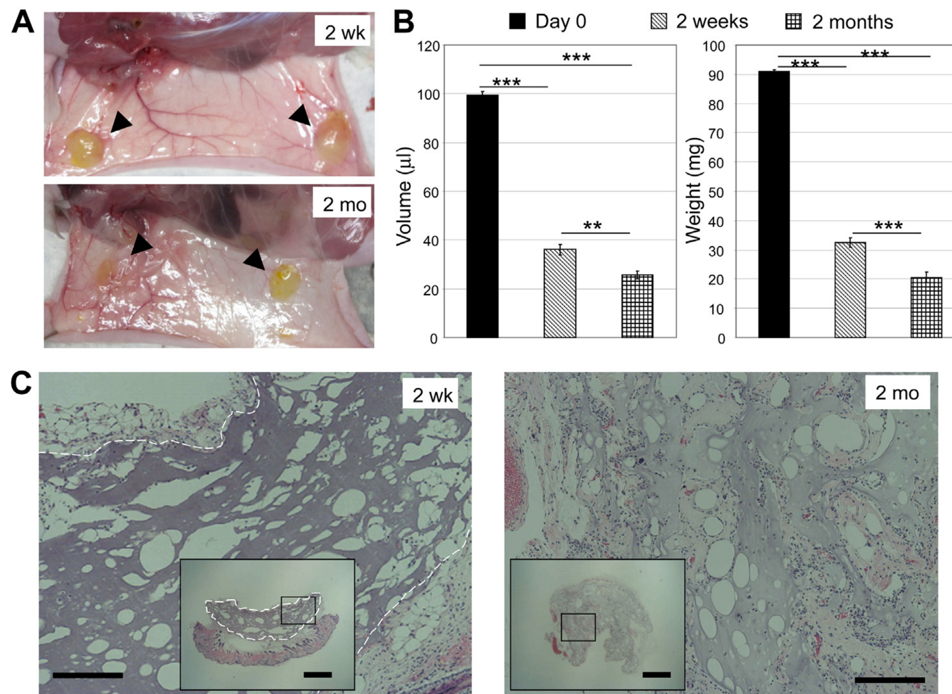

Figure 7. Adipocytes/hydrogel mixture in immunodeficient mice. Hydrogel-enclosed human mature adipocytes were delivered to athymic nude mice. Four sites of the subcutaneous skin received 0.1 mL of the mixture by injection; (A) 2 weeks (upper) and 2 months after injection. Delivered materials are indicated by arrowheads. (B) Weight (mg) and volume (µL) measurement. The mixtures at day 0 ($n = 8$), 2 weeks ($n = 8$) and 2 months ($n = 9$) were analyzed. $^{**}P < 0.01$, $^{***}P < 0.001$. (C) Hematoxylin and eosin staining. The mixture was harvested with host skin tissue 2 weeks after injection (left). Junction area between the mixture and host tissue is demarcated by white dotted lines. The mixture was harvested without host skin tissue 2 months after injection (right). Inlets are analyzed areas with lower magnification. Scale bar, 200 µm; inlet, 1000 µm.

de-differentiated adipocytes proliferate in a 3-dimensional collagen gel matrix *in vitro* [36,37]. Our studies with BrdU labeling and Ki67 staining suggest that mature mouse and human adipocytes have proliferative potential *in vitro* (Figure 4) [22]. Although indications that adipocytes proliferate *in vivo* have previously been reported [18,33], the proliferation of mature adipocytes *in vivo* is controversial.

Our analysis of abdominal subcutaneous adipocytes shows a positive correlation between BMI and the size of mature adipocytes (Figure 1). Consistent with this finding, other reports have suggested that the size of mature adipocytes is closely related to BMI [38–41]. BMI is determined by dividing one's weight in kilograms by the square of their height in meters. People are classified as underweight (<18.5), normal (18.5–24.9), overweight (25.0–29.9) and obese (≥30.0), according to BMI. Higher BMI is associated with increased all-cause mortality in adults [42]. Positive correlation between BMI and cytokine expression, such as nesfatin-1, and TNF-α has previously been shown [43,44]. Although all adipocytes are morphologically similar, their characteristics vary, depending on location. The size of adipocytes within a single patient (or animal) also varies, depending on the location of the fat tissue [39–41]. Adipocytes in WAT are unilocular cells, whereas those in BAT are multilocular cells [1,13,45]. Gene expression in visceral white adipocytes is different from that of subcutaneous adipocytes [46–49]. A comparative analysis of these diverse adipocyte types and their response to hypoxia or H-R injury is crucial to our understanding of adipocyte physiology. Additionally, the study of adipocyte behavior in disease conditions such as diabetes is important to our overall understanding of adipocyte function.

In summary, we showed that human mature adipocytes can be cultured *in vitro* by enclosing them in a 3-dimensional matrix. Our data suggest that human mature adipocytes are functionally active cells that respond to environmental changes such as hypoxia and H-R.

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