島正洋

第48回日本婦人科腫瘍学会（つくば国際会議場、茨城）2010年7月8-10日

妊娠中の子宮頸部細胞診における日母分類、ベセスダシステムおよびHPVスクリーニングの比較．三浦清徳、山崎健太郎、池本理恵、三浦生子、嶋田貴子、濱口大輔、小寺宏平、藤下晃、鮫島哲郎、村上誠、中山大介、吉浦孝一郎、増崎英明

第34回日本口蓋裂学会総会・学術集会 2010年5月27日（木）～28日（金），北とぴあ，東京

シンポジウムIテーマ：口唇裂・口蓋裂の分子遺伝学研究 –これまでの研究成果とこれからの原因追求 -S Ⅰ-基調講演: 比較的ありふれた病気（sub-common disease）としての口唇列・口蓋裂．吉浦孝一郎

第106回 日本精神神経学会学術総会 2010年5月20日（木）～22日（土）広島国際会議場, 広島

2-F-18: 統合失調症および自閉症一卵性双生児不一致例におけるゲノム構造変化の検証．小野慎治，今村　明，橋田あおい，黒滝直弘，田崎真也，小澤　寛樹，吉浦孝一郎

第110回日本外科学会総会 2010年4月8日（木）～10日（土），名古屋国際会議場，名古屋

PD-9-1: 乳腺乳頭状腫瘍の臨床病理学的特徴と細胞遺伝学的プロファイル．及川将弘, 吉浦孝一郎, 矢野 洋, 安倍邦子, 林徳真吉, 永安　武

国際学会

2010/10/10-14 20th ISUOG World Congress: (Prague, Czech Republic)

A case of mesenchymal diaplasia. Miura K, Yamasaki K, Miura S, Nakayama D, Yoshiura K, Nakayama M, Masuzaki H

The American Society of Human Genetics, 59th Annual Meeting

Washigton D.C., Baltimore, November 2-6, 2010

1147/T: Frequency of 27-bp deletion mutation, another earwax determinant, in ABCC11 among the Japanese population.(1447) (5:00PM-6:00PM on Thu) **Author(s):** A. Yamada, Y. Hori, Y. Ono, N. Matsuda, D. Starenki, N. Sosonkina, K. Yoshiura, T. Ohta, N. Niikawa

2219/F: Re-sequencing analysis of candidate region for a neurodegenerative disorder by massively parallel sequencing. T. Kaname, A. Tsujino, K. Yanagi, K. Hayashi, M. Tsukahara, K. Fujimori, I. Kikuzato, M. Teruya, Y. Imada, M. Nezuo, S. Yano, Y. Sato, Y. Miwa, T. Niikawa, K. Yoshiura, K. Naritomi

H．知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

Beckwith-Wiedemann 症候群のゲノムワイドメチル化解析

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研究要旨

本研究計画では、Beckwith-Wiedemann 症候群を、エピゲノム異常の観点から網羅的に精査し、エピフェノタイプ（エピジェネティックな状態）と症状との関連を抽出し、今後の診断基準等に役立てる事を目的とする。解析は、研究者らが独自に確立したヒトインプリンティング遺伝子の制御領域を含む 32 箇所の DNA メチル化状態を網羅的に解析する系と、illumina 社 infinium アッセイによるゲノムワイドメチル化解析を行った。
Beckwith-Wiedemann 症候群の診断および解析に、我々が独自に確立した DMR の DNA メチル化スクリーニング系は有用であった。全ゲノム網羅的な DNA メチル化異常のスクリーニングを行ったところ、正常対照群と比較して顕著な DNA メチル化異常は存在しないが、今後更に統計的手法を用いた解析が必要と考えられた。

A．研究目的

本研究計画では、研究代表者副島らの収集した Beckwith-Wiedemann 症候群患者検体および病歴等の臨床情報を用い、これらの疾患をエピゲノム異常の観点から、従来必ずしも解析されていなかった領域も含めて網羅して精査し、エピフェノタイプ（エピジェネティックな状態）と症状との関連を抽出し、今後の診断基準等に役立てる事を目的とする。

B．研究方法

本研究で解析対象とする症例は、ゲノムインプリンティングの破綻が疑われ、その直接の原因として、遺伝子変異あるいは DNA メチル化異常が疑われる。研究代表者らは、すでにヒトインプリンティング遺伝子の制御領域を含む 32 箇所の DNA メチル化状態を網羅的に解析する系を確立している。この解析系を用い、BWS 症例検体の網羅的 DNA メチル化解析を行う。

C．研究結果

初年度にBWS症例検体57例に対し、上記の Bio-COBRA 法を用い、ヒトDMR23か所の網羅的解析を行った。その結果、末梢血ゲノムDNAを解析した27例のうち20例については既知 BWS 変異が同定されており（pUPD11症例が5例、LIT-DMR低メチル化症例が 10 例、H19-DMR 高メチル化症例が2例、KIP2変異症例が3例）、今回実施したbio-COBRA解析の結果は既知の分子診断結果と一致した。pUPD11症例（5例）において、H19-DMR高メチル化とLIT1-DMR低メチル化が検出され、他のDMRにおけるメチル化異常はなかった。LIT-DMR低メチル化症例（10例）については、LIT1-DMR低メチル化に加えて、1例でMCTS2-DMR低メチル化、別の一例で MEG3-DMR 高メチル化傾向が検出された。H19-DMR高メチル化症例（2例）については、H19-DMR高メチル化に加えてH19-DMRの制御下にあることが知られている IGF2遺伝子座のDMR（DMR0, DMR2)における高メチル化傾向も検出されたが、それら以外のDMRではメチル化異常は無かった。原因未同定の7症例のうちの 1 症例で、14 番染色体上のIG-DMR および MEG3-DMR における顕著な高メチル化傾向（メチル化レベル>90%）が見られ BWS 診断規準および pUPD14 症候群診断規準（胸郭低形成（ベル型）、ロート胸など）の両方を満たしていることが判明した。illumina社 infinium アッセイによるゲノムワイドメチル化解析により，プロモーター領域を中心とした 27,000 か所の DNA メチル化レベルを定量的に測定することが可能である。BWS症例についてゲノムワイドなメチル化解析を実施することで、DMR メチル化異常 BWS 症例におけるエピゲノム異常部位の特異性が評価できること、未知のエピゲノム異常が同定できることなどが期待される。そこで5例のBWS症例についてゲノムワイドメチル化データを予備的に取得し、データ解析を行ったているが、現在のところ、BWS症例で特異的な、あるいは、非特異的であっても、正常対照群と比較して有意にDNAメチル化状態の乱れが認められる領域は見つかっていない。

D．考察

この研究計画に伴って、我々が独自に確立した複数DMRのDNAメチル化スクリーニングは、既存の手法と矛盾なく診断が可能であると共に、定型的診断領域以外のDMRにDNAメチル化異常を認め、片親性ダイソミー等の異常を見出す事が稀ならずあった。既知の疾患関連領域に

明確な DNA メチル化異常を認めない場合はもちろん、既知関連領域に DNA メチル化異常を認める場合も、網羅的な DNA メチル化解析はより正確な診断の為に有用であると考えられた。

illumina 社 infinium アッセイによる解析は、今後は統計的な処理を進め、BWS 症例と正常対照群を比較し、DNA メチル化の非特異的な変動の有無を詳細に検討する。

E．結論

Beckwith-Wiedemann 症候群の診断および解析に、我々が独自に確立した DMR の DNA メチル化スクリーニング系は有用であった。全ゲノム網羅的な DNA メチル化異常のスクリーニングを行ったところ、正常対照群と比較して顕著な DNA メチル化異常は存在しないが、今後更に統計的手法を用いた解析が必要と考えられた。

F．健康危険情報

特記事項なし。

G．研究発表

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[総説（和文）]

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[特別講演・シンポジウム]

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H．知的財産権の出願・登録状況　　なし

III. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
副島英伸, 城圭一郎, 中尾光善	23章エピジェネティクスとヒト疾患	D. アリス・T. ジェニュワイン・D. ラインバーグ共編 堀越正美監訳.	エピジェネティクス	培風館	東京	2010	505-528

雑誌

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IV. 研究成果の刊行物・別冊

Pediatr Blood Cancer 2010;55:733–735

BRIEF REPORT
Acute Megakaryocytic Leukemia (AMKL,FAB;M7) With Beckwith–Wiedemann Syndrome

Shohei Yamamoto, MD,[1,*] Daisuke Toyama, MD,[1] Hitomi Yatsuki, MD,[2] Ken Higashimoto, MD,[2] Hidenobu Soejima, MD,[2] and Keiichi Isoyama, MD[1]

Beckwith–Wiedemann syndrome (BWS) is characterized by an accumulation of multiple congenital anomalies. Although patients with BWS are known to have a higher incidence of embryonal tumors, there has been no reports associated with acute leukemia. This report describes the case of a patient with BWS who developed Acute Megakaryocytic Leukemia (AMKL,FAB;M7). Because most patients with BWS present gigantism, the therapy-related toxicity of chemotherapy can be a very serious problem. This patient exhibited no therapy-related toxicity after chemotherapy, suggesting that acute leukemia with BWS may not require a reduction in dosage. Pediatr Blood Cancer. 2010;55:733–735. © 2010 Wiley-Liss, Inc.

Key words: AMKL; Beckwith–Wiedemann Syndrome; chemotherapy; gigantism

INTRODUCTION

Beckwith–Wiedemann syndrome (BWS) is characterized by an accumulation of multiple congenital anomalies. Exophalos, macroglossia, and giantism are considered the most common manifestations [1,2]. Patients with BWS also have a higher incidence of embryonal tumors, such as Wilms tumors [1,2]. This report presents the case of a patient with BWS who developed Acute Megakaryocytic Leukemia (AMKL,FAB:M7).

CASE REPORT

An 1-year- and-3-month-old male presented with a continuous fever. His white blood cell (WBC) count was elevated. The patient was born at 39 weeks of gestation by cesarean section because of fetal distress. At birth, the patient weighed 3,762 g (97th percentile) and had a height of 52 cm (90th percentile). The patient had an omphalocele, macroglossia, bilateral linear creases on the ear lobes, hypoglycemia, and patent ductus arteriosus. As a result, the patient was diagnosed with BWS.

Upon admission the patient's weight and height were 13.2 kg (97th percentile) and 85.8 cm (97th percentile), respectively. The patient displayed cervical lymphadenopathy and hepatosplenomegaly, but kidneys were normal upon an abdominal ultrasound. The patient's WBC count was $119.0 \times 10^3/\mu l$, with 82% leukemic blasts. The hemoglobin level was 6.0 g/dl and the platelet count was $2.2 \times 10^4/\mu l$. Serum levels of lactate dehydrogenase (LDH) were elevated (3,587 IU/L; normal range, 240–530 IU/L). Bone marrow aspiration revealed a normocellular marrow with 85% leukemic blasts (Fig. 1). The leukemic blasts were negative for myeloperoxidase, naphthol AS-D chloroacetate and α-naphthyl butyrate. Surface marker analysis using CD45 blast gating showed the leukemic blasts to be positive for CD4, CD33, CD36, CD41, CD42b, and CD61, thus indicating megakaryocytic origin. Chromosomal analysis showed monosomy 7 with 45, XY, -7, der(10)t(7;10)(q11;p11)ins(10;?)(p11;?). The patient was diagnosed with AMKL. In the patient's leukemic and somatic cells, no methylation of the imprinted domain was observed at the 11p15.5 region (namely, no methylation of DMR-LIT1 and H19-DMR). In addition, no paternal uniparental disomy (patUPD) or CDKNIC mutations were seen. The patient was treated with a low dose cytosine arabinoside (AraC) for cytoreduction. Because there is no report of BWS associated with acute leukemia and because the patient was large for his age, the possibility for therapy-related toxicity due to full dosage induction therapy was a concern. Therefore, the patient received induction therapy according to protocol AML99-Down (AraC 100 mg/m^2 for 7 days; pirarubicin 25 mg/m^2 on days 1 and 2; etoposide 150 mg/m^2 on days 3, 4, and 5), which were lower doses than for conventional induction therapy for patients without Down syndrome. However, the patient failed to achieve remission. He thereafter received full dose chemotherapy according to protocol AML99-inductionC (AraC 500 mg/m^2 on days 1–3 and on days 8–10; idarubicin 8 mg/m^2 on days 1–3; etoposide 200 mg/m^2 on days 8–10). The patient did not experience any therapy-related toxicity thereafter and a complete remission was achieved. He received allogeneic cord blood transplantation (CBT) upon the first remission after three courses of consolidation therapies.

The preparative regimen included busulfan (4.8 mg/kg intravenously × 4 days), etoposide (60 mg/kg intravenously × 1 day), cyclophosphamide (60 mg/kg intravenously × 2 days). The donor was an unrelated female CB (8.0×10^7 nucleated cell/kg) mismatched at 1 HLA loci (HLA-B antigen mismatch). The patient received 0.03 mg/kg tacrolimus and short-term methotrexate (15 mg/m^2 on day1, 10 mg/m^2 on days 3, 6, and 11) for prophylaxis of acute graft-versus-host disease (aGVHD). On day 21, Grade III aGVHD (skin, stage 3; liver, stage 0; gut, stage 3) developed. Therefore, the patient received daily intravenous administration of 2 mg/kg prednisolone (PSL). The skin and gut symptoms resolved

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Conflict of Interest: The authors declare no conflict of interest.

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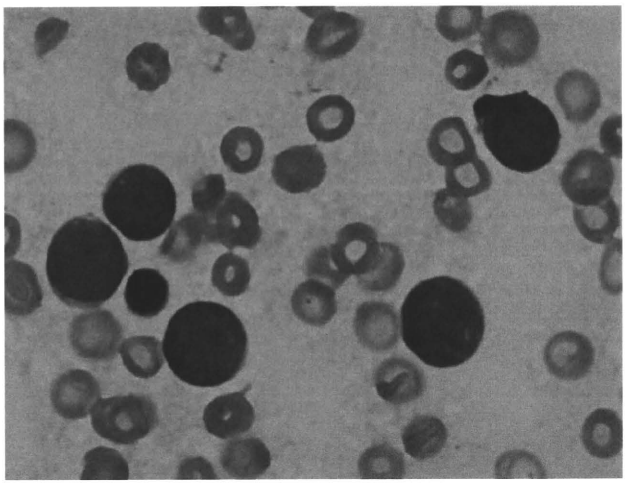

Fig. 1. A bone marrow smear showed small size blasts with high a nucleus-cytoplasm ratio, round nuclei with fine chromatin formation and a few nucleoli. The findings are negative for peroxidase and esterase staining. (Wright-Giemsa staining × 1,000).

after the administration of PSL. The white blood cell count exceeded 1,000/μl on day 36 after CBT. A bone marrow sample obtained on day 45 post-transplant showed 50% leukemic blasts. A chromosomal study showed the same results as the initial diagnosis. FISH analysis revealed 25% donor cells. Tacrolimus treatment was discontinued. However, the skin lesions for aGVHD progressed and intravenous administration of tacrolimus and PSL were restarted. The skin results were stable at stages 2–3 until day 90 and the WBC count was maintained at 2,000–3,000/μl. Thereafter, the skin results gradually resolved, and the WBC count increased. The patient was treated with low dose AraC. However, the patient's general condition did not improve and he died 10 months after CBT from progression disease.

DISCUSSION

Beckwith–Wiedemann syndrome (BWS) is associated with multiple congenital anomalies, exophalos, macroglossia and giantism [1]. Other symptoms include neonatal hypoglycemia, ear creases, hemihypertrophy and cardiac defects [2]. BWS also has a higher incidence of embryonal tumors, such as Wilms tumors [3]. Most cases are sporadic.

BWS is caused by dysregulation of imprinted growth regulatory genes within the 11p15.5 region [4]. The 11p15.5 region contains two independent imprinted domains, IGF2/H19 and KIP2/LIT1. Imprinted genes within each domain are regulated by the imprinting control region (ICR), which, in this case, is either H19-DMR or DMR-LIT1 [5,6]. BWS have been identified DMR-LIT1 loss of DNA methylation, H19-DMR DNA hypermethylation, paternal uniparental disomy (patUPD), and CDKN1C mutations [5,6]. The cause is unknown in approximately 30% of all Japanese BWS patients [6]. In the patient's somatic cells, there was no methylation of DMR-LIT1 and H19-DMR, and there were no patUPD and CDKNIC mutations. As a result, the etiology of BWS remained unclear. Further investigations will therefore be necessary to understand whether the different frequencies of epigenetic and genetic alterations and due to DNA polymorphisms.

BWS predisposes patients to develop embryonal tumors, especially Wilms tumors [2], but there have been no reports of a patient with BWS and acute leukemia. Wilms tumor development in BWS has a strong association with H19-DMR hypermethylation, DMR-LIT1 loss of methylation, and patUPD [7,8]. However, in the patient's leukemic cells, no methylation of DMR-LIT1 or H19-DMR was observed, and no patUPD or CDKNIC mutations were seen either. We did not perform Comparative Genomic Hybridization (CGH) because the patient did not exhibit any methylation of DMR-LIT1 and H19-DMR. To analyze patUPD, we used DNA polymorphic markers for 11p15.5, including tetranucleotide repeats in the tyrosine hydroxylase (TH) and D11S1997, trinucleotide repeats in the D11S2326, dinucleotide repeats in the D11S1318. These were all both paternal and maternal alleles. Therefore, there were no defects at 11p15.5.

The leukemic cells had monosomy 7 with der(10)t(7;10)(q11;p11). This is an atypical association with AMKL. This unbalanced translocation may have contributed to the development of AMKL.

There have so far been no reports of BWS patients who develop acute leukemia. In addition, there is no data indicating optimal therapy that is optimal or tolerable.

The weight and height of the current patient corresponded to the normal values for a 3-year-old and 2-year-old child, respectively. The patient was over 20% of the normal height for his age because of the obesity associated with BWS, and this overgrowth may be linked to increased levels of growth hormones [9], the BWS-associated overgrowth was thought to be balanced. We did not check the patient's insulin-like growth factor levels, which may have been elevated [9]. The patient did not experience any therapy-related toxicity after AML99-inductionC and consolidation therapies. Therefore, no reduction in the dosage of chemotherapy for acute leukemia with BWS was deemed necessary.

The treatment results for children without Down syndrome who have AMKL have been poor [10,11]. In particular, the outcome is extremely poor in children who fail to attain remission or who experience a disease relapse [12]. The current patient underwent allogeneic stem cell transplantation (allo SCT) during the first CR because monosomy 7 was detected.

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Original Article

Organotypic culture of human bone marrow adipose tissue

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The precise role of bone marrow adipose tissue (BMAT) in the marrow remains unknown. The purpose of the present study was therefore to describe a novel method for studying BMAT using 3-D collagen gel culture of BMAT fragments, immunohistochemistry, ELISA and real-time reverse transcription–polymerase chain reaction. Mature adipocytes and CD45+ leukocytes were retained for >3 weeks. Bone marrow stromal cells (BMSC) including a small number of lipid-laden preadipocytes and CD44+/CD105+ mesenchymal stem cell (MSC)-like cells, developed from BMAT. Dexamethasone (10 μmol/L), but not insulin (20 mU/mL), significantly increased the number of preadipocytes. Dexamethasone and insulin also promoted leptin production and gene expression in BMAT. Adiponectin production by BMAT was <0.8 ng/mL under all culture conditions. Dexamethasone promoted adiponectin gene expression, while insulin inhibited it. This finding suggests that dexamethasone, but not insulin, may serve as a powerful adipogenic factor for BMAT, in which adiponectin protein secretion is normally very low, and that BMAT may exhibit a different phenotype from that of the visceral and subcutaneous adipose tissues. BMAT–osteoblast interactions were also examined, and it was found that osteoblasts inhibited the development of BMSC and reduced leptin production, while BMAT inhibited the growth and differentiation of osteoblasts. The present novel method proved to be useful for the study of BMAT biology.

Key words: adipocytes, adipokine, bone marrow adipose tissue, bone marrow stromal cells, dexamethasone, hematopoietic cells, mesenchymal stem cells, organotypic culture, osteoblasts

Bone marrow adipose tissue (BMAT) consists of multiple cell types including mature adipocytes and hematopoietic cells. BMAT has been suggested to function in many aspects of marrow homeostasis such as (i) simply occupying excess space in the marrow cavity; (ii) systemic lipid metabolism; (iii) serving as a localized energy reservoir; and (iv) the regulation of hematopoiesis, osteogenesis and osteoclastogenesis.[1] The precise roles of BMAT in bone marrow homeostasis, however, remain unknown. To investigate the function of the multiple cell types containing BMAT, an appropriate culture system of BMAT seems to be required, but the method has not been established. One of the major reasons for the lack of an appropriate culture system is the difficulty in culturing BMAT, which contains a large number of lipid droplet-embracing mature adipocytes that do not attach to the surface of the culture dish and that are buoyant in the culture medium.

To investigate the roles of BMAT in bone marrow homeostasis, we developed a novel culture system using BMAT fragments embedded in a 3-D collagen gel that was able to easily trap buoyant BMAT. This novel method was based on our previously reported culture method of subcutaneous adipose tissue.[2,3] BMAT *in vivo* is adjacent to bone trabecular surface-lining osteoblasts, suggesting their critical interaction. Thus, we also demonstrated the interaction between BMAT and osteoblasts *in vitro*, as an application of this novel method.

MATERIALS AND METHODS

Culture system

All experimental procedures outlined in the present study were pre-approved by the ethics committee of Saga University and were conducted in accordance with the ethics guidelines of this university. BMAT was obtained from the femoral bone marrow of four female patients (age range,

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60–71 years; mean age, 65.5 ± 5.8 years) who had osteoarthritis of the hip and underwent orthopaedic surgery for arthroplasty at Saga University. Fragmented bone-containing materials (3–10 mL) were placed in Petri dishes containing 10 mL PBS for 10 min. Once the bone fragments had precipitated to the bottom of the dish, the BMAT fragments that remained floating in the PBS were collected. These fragments were then minced within approximately 0.5 mm diameter. The fragments contained numerous mature adipocytes and a small number of mononuclear blood cells that had leukocyte morphology. The mononuclear blood cells were not megakaryocytes or normoblasts, because the starting materials were obtained from the fatty bone marrow. A total of 0.1 mL BMAT fragments was then embedded in 1.0 mL type I collagen gel solution (Nitta Gelatin, Osaka, Japan), and cultured in Ham's F-12 medium supplemented with 10% newborn calf serum and 50 μg/mL gentamicin (Fig. 1a). The medium was exchanged for fresh medium every 2 days. In some cases, 10 μmol/L dexamethasone (Sigma-Aldrich, St Louis, MO, USA)[4] and 20 mU/mL insulin (Sigma-Aldrich)[5] were also added to the medium upon each exchange.

Histology and morphometry

For histology and morphometry, we fixed the cellular layer gel with 5% formalin and routinely processed the gel to paraffin. We then deparaffinized the sections and stained the cells with HE. The sections were then observed on light microscopy. To detect both the mature adipocytes and preadipocytes, we carried out oil red O staining on samples fixed with osmic acid as previously described.[6,7] We called the spindle-shaped cell types that were found to develop from the BMAT fragments bone marrow stromal cells (BMSC). In the BMSC population, the cells that were S-100 protein positive and demonstrated fine lipid droplets were designated preadipocytes. We then counted the total number of BMSC using a ×20 objective in five randomly chosen areas surrounding the BMAT fragments identified in sections stained for either histochemistry or immunohistochemistry. The percentage of preadipocytes was calculated using the formula (no. preadipocytes)/(total no. BMSC) ×100 (%). Because hematopoietic cells within BMAT demonstrated mononuclear leukocyte morphology in culture, we also examined the expression of the leukocyte markers as described in the following section. Finally, we estimated the formation of bone, cartilage and muscle tissues, which may be organized by BMSC, on the basis of their specific morphology,[8] using HE staining sections of the culture assembly.

Immunohistochemistry and immunofluorescence

We used the rabbit polyclonal S-100 protein antibody (DakoCytomation, Glostrup, Denmark) to detect mature adipocytes and preadipocytes. To identify leukocytes, we used mouse monoclonal antibodies directed against the leukocyte common antigen CD45 (Nichirei, Tokyo, Japan), neutrophil elastase, CD20, CD79a, CD3,[9] PG-M1 (DakoCytomation), and CD163 (NovoCastra Laboratories, Newcastle upon Tyne, UK). Deparaffinized sections were immunostained using the avidin–biotin complex immunoperoxidase (ABC) method as described previously.[10] It has been shown previously that adipose tissue generally contains CD44+/CD105+ mesenchymal stem cells (MSC).[11,12] To detect MSC-like cells, we used mouse monoclonal CD44 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and CD105 antibodies (NovoCastra Laboratories). To confirm the co-localization of CD105 and CD44, we carried out double color immunofluorescence on deparaffinized sections using fluorescein isothiocyanate- or rhodamine-conjugated avidin (Molecular Probes, Eugene, OR, USA) as described previously.[2,13] We also carried out double immunohistochemistry using bromodeoxyuridine (BrdU) and each of the antibodies listed here as described previously.[10]

Cell proliferation

We examined cell growth in the culture assembly at 1 week using BrdU (Cell Proliferation Kit, Amersham, Arlington Heights, IL, USA) immunohistochemistry. Briefly, the cells were incubated with 3 μg/mL BrdU after 48 h in culture.[14] A total of 100 cells within BMAT in randomly chosen high-power fields (x20 objective) were counted and the percentage of BrdU intake in both the S-100+ mature adipocytes containing large lipid droplets and the CD45+ leukocytes was calculated. The percentage of BrdU-positive preadipocytes and non-lipid containing BMSC in 100 BMSC was also calculated using this method.

Real-time reverse transcription–polymerase chain reaction

We investigated the expression of peroxisome proliferator-activated receptor (PPAR)γ, adiponectin and leptin using real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was extracted from BMAT after 1 week in culture using Isogen (Nippon Gene, Tokyo, Japan). To increase the purity of the complementary DNA (cDNA), total RNA was re-extracted using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany). PCR was then performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR was undertaken in a total volume of 20 μL containing Power SYBR green PCR Master Mix (Applied Biosystems) and Quantitect Primers for PPARγ (Hs_PPAG_1_SG), leptin

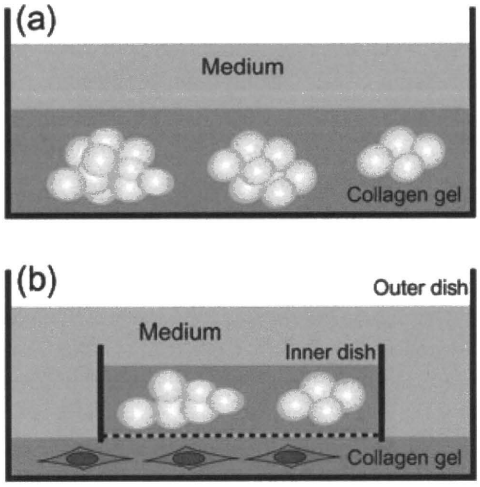

Figure 1 Bone marrow adipose tissue (BMAT) organotypic culture and co-culture systems of BMAT fragments and osteoblasts. (**a**) A total of 0.1 mL of BMAT fragments obtained from femoral bone marrow is embedded in 1.0 mL type I collagen gel solution. (**b**) BMAT fragments and osteoblasts are embedded in collagen gel in inner and outer dishes, respectively. These two layers are completely separated by nitrocellulose membrane.

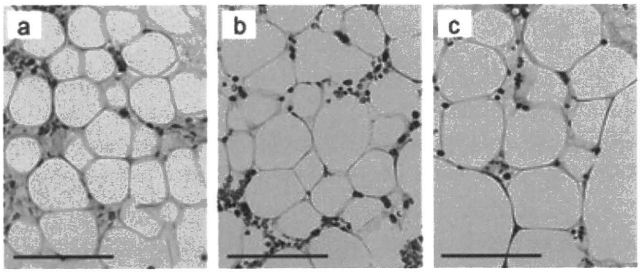

Figure 2 Histology of the human bone marrow adipose tissue (BMAT)-organotypic cultures. (**a**) BMAT immediately after being embedded in the collagen gel (day 0) exhibits mature adipocytes, mononuclear blood cells and erythrocytes, but not spindle-shaped cells. Note that the mature adipocytes contain a large single lipid droplet. At (**b**) 1 week and (**c**) 3 weeks in culture, mature adipocytes and mononuclear blood cells are maintained within BMAT. HE staining. Bars, 100 μm.

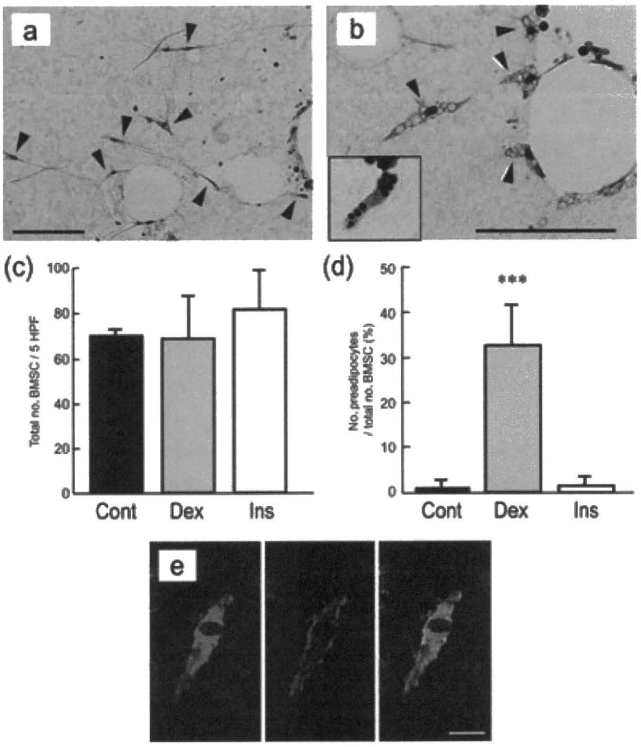

Figure 3 Development of bone marrow stromal cells (BMSC) around bone marrow adipose tissue (BMAT) after 1 week in culture. (**a**) Non-lipid containing BMSC (arrowheads) develop actively around BMAT. (**b**) Addition of 10 μmol/L of adipogenic agent dexamethasone extensively increases the number of preadipocytes (arrowheads) containing oil red O-positive lipid droplets (inset). Bars, 100 μm. (**c**) Dexamethasone and insulin (20 mU/mL) do not affect the total number of BMSC that develop from the BMAT fragments. (**d**) Dexamethasone significantly increases the percentage of preadipocytes among BMSC (***$P < 0.001$ vs control or insulin), while insulin does not. (**e**) Immunofluorescence shows that some BMSC co-express CD105 (green) and CD44 (red). CD105 and CD44 are clearly merged (right panel; bar, 20 μm). The CD105+/CD44+ mesenchymal stem cell-like cells are detected at a rate of 2.8 ± 1.3% in BMSC. Cont, control; Dex, dexamethasone; Ins, insulin. HPF, high-power field.

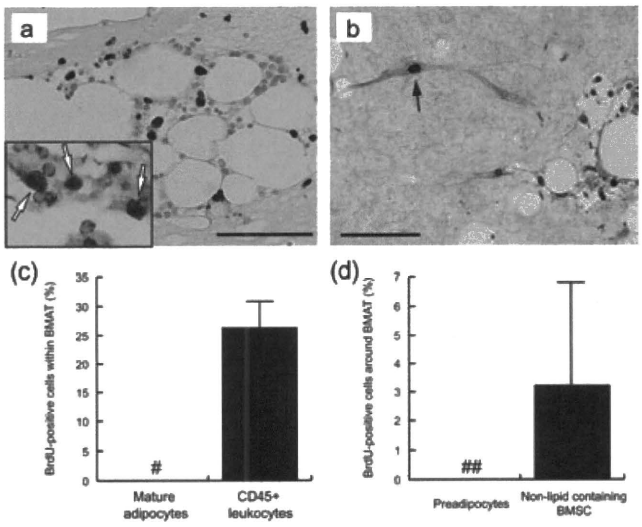

Figure 4 Bromodeoxyuridine (BrdU) immunohistochemistry of (**a**) bone marrow adipose tissue (BMAT) and (**b**) bone marrow stromal cells (BMSC), and BrdU uptake rates of (**c**) mature adipocytes and CD45+ leukocytes, and (**d**) preadipocytes and non-lipid-containing BMSC after 1 week in culture. (**a**) BrdU uptake (black) is detected only in mononuclear blood cells within BMAT, and is not detected in the mature adipocytes. Inset (**a**), the CD45+ (red) leukocytes (arrows) contain BrdU (black). (**b**) BrdU uptake is detected in BMSC (arrow). Bars, 100 μm. (**c**) CD45+ leukocytes exhibit BrdU uptake (26.4 ± 4.7%), whereas mature adipocytes (#) do not. (**d**) Non-lipid-containing BMSC exhibit BrdU intake (3.2 ± 3.7%), while preadipocytes (##) do not.

(Hs_LEP_1_SG), adiponectin (Hs_ADIPOQ_1_SG) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs_GAPDH_2_SG; Qiagen). The expression of each gene was normalized to that of GAPDH.

Adipokine production

We measured adiponectin and leptin levels in the supernatant after 1 week in culture using the human ELISA kits of adiponectin (assay sensitivity: 50 pg/mL; AdipoGen, Seoul, South Korea) and leptin (assay sensitivity: 7.8 pg/mL; R&D Systems, Minneapolis, MN, USA). The adiponectin kit detected its total form.

Effects of soluble factors on adipokine production and gene expression

We next examined the effects of the following factors on adipokine production and gene expression: (i) adipogenic factors of dexamethasone and insulin; and (ii) inflammation-related agents of tumor necrosis factor-α (TNF-α; R&D Systems) and lipopolysaccharide (LPS; *Escherichia coli* 0127 B8, Sigma-Aldrich). One week cultures were stimulated with 10 μmol/L dexamethasone,[4] 20 mU/mL insulin,[5] 2 nmol/L TNF-α[15] or 10 μg/mL LPS[16] for 48 h and analyzed using ELISA and real-time RT-PCR as described in the previous sections.

BMAT-osteoblast interactions

We established co-cultures of the BMAT fragments and osteoblasts (Fig. 1b) as follows. Type I collagen gel (1 mL; Nitta Gelatin) containing 1×10^6 MC3T3-E1 osteoblasts (ATCC, Manassas, VA, USA)[17] was added to a six-well plate (outer dish), and the gel (1 mL) containing 0.1 mL BMAT fragments was poured into a 30 mm diameter dish (inner dish), the bottom of which contained nitrocellulose membrane (Millicell-CM, Millipore, Bedford, MA, USA). The inner dish was then placed on the outer dish and medium added to both dishes. In this system, cells were fed sufficient culture medium in both the inner and outer dishes due to the permeability of the nitrocellulose membrane. The BMAT fragments or osteoblasts cultured alone in the gel served as the controls. Cells cultured in this manner were analyzed using the methods described in the previous section. We also examined the gene expression levels of alkaline phosphatase (ALP), type I collagen, and osteocalcin in the osteoblasts. For gene expression analysis, real-time RT-PCR was undertaken in a total volume of 20 μL containing Power SYBR green PCR Master Mix (Applied Biosystems) and the Quantitect Primer pairs for ALP (Mm_Akp2-1_SG), type I collagen (Mm_Col1a1-1_SG), osteocalcin (Mm_Bglap1-1_SG) or β-actin (Mm_Actb_2_SG) (Qiagen). The expression of each gene was normalized to that of β-actin.

Statistical analysis

Statistical differences between the data obtained in 4–10 independent experiments were analyzed using Student's *t*-test. Values are presented as mean ± SD. $P < 0.05$ was considered significant.

RESULTS

BMAT-organotypic culture

Immediately after being embedded in the gel, the BMAT fragments exhibited numerous mature adipocytes and a small number of mononuclear blood cells and erythrocytes (Fig. 2a). Viable mature adipocytes and mononuclear blood cells were maintained for at least 3 weeks in culture (Fig. 2b,c). The central portions of the fragments demonstrated no significant changes. At 1 week in culture, mononuclear blood cells consisted of B and T lymphocytes, and macrophages (approx. 23.4%, 33.3%, and 36.2%, respectively). In contrast, the peripheral zones of the fragments underwent numerous prominent changes. After 1 week in culture, BMSC (70.0 ± 3.6 cells) developed at the peripheral zones (Fig. 3a,c). In these BMSC the proportion of lipid-containing preadipocytes was 0.75 ± 0.96%. The number of BMSC increased with culture term (3 weeks in culture: 86.0 ± 10.4, $P < 0.05$, vs 1 week; 4 weeks in culture: 93.5 ± 9.5, $P < 0.005$, vs 1 week). The adipogenic factors dexamethasone and insulin had no significant effects on the morphology of mature adipocytes. In addition, these agents failed to affect the total number of BMSC (dexamethasone: 68.8 ± 18.0 cells, $P = 0.89$, vs control; and insulin: 81.3 ± 17.8 cells, $P = 0.26$, vs control; Fig. 3c). But dexamethasone significantly increased the number of preadipocytes (32.0 ± 7.5%, $P < 0.001$, vs control; Fig. 3b,d), a result that was not observed following treatment with insulin (Fig. 3d). Among the BMSC, a few CD44+/CD105+ MSC-like cells (2.8 ± 1.3%) were also observed (Fig. 3e). We did not detect any bone, cartilage or muscle tissues in the culture assembly, based on their specific morphology.[8]

Cell proliferation

Within the BMAT fragments, mature adipocytes demonstrated no BrdU uptake (Fig. 4a,c), while the CD45+

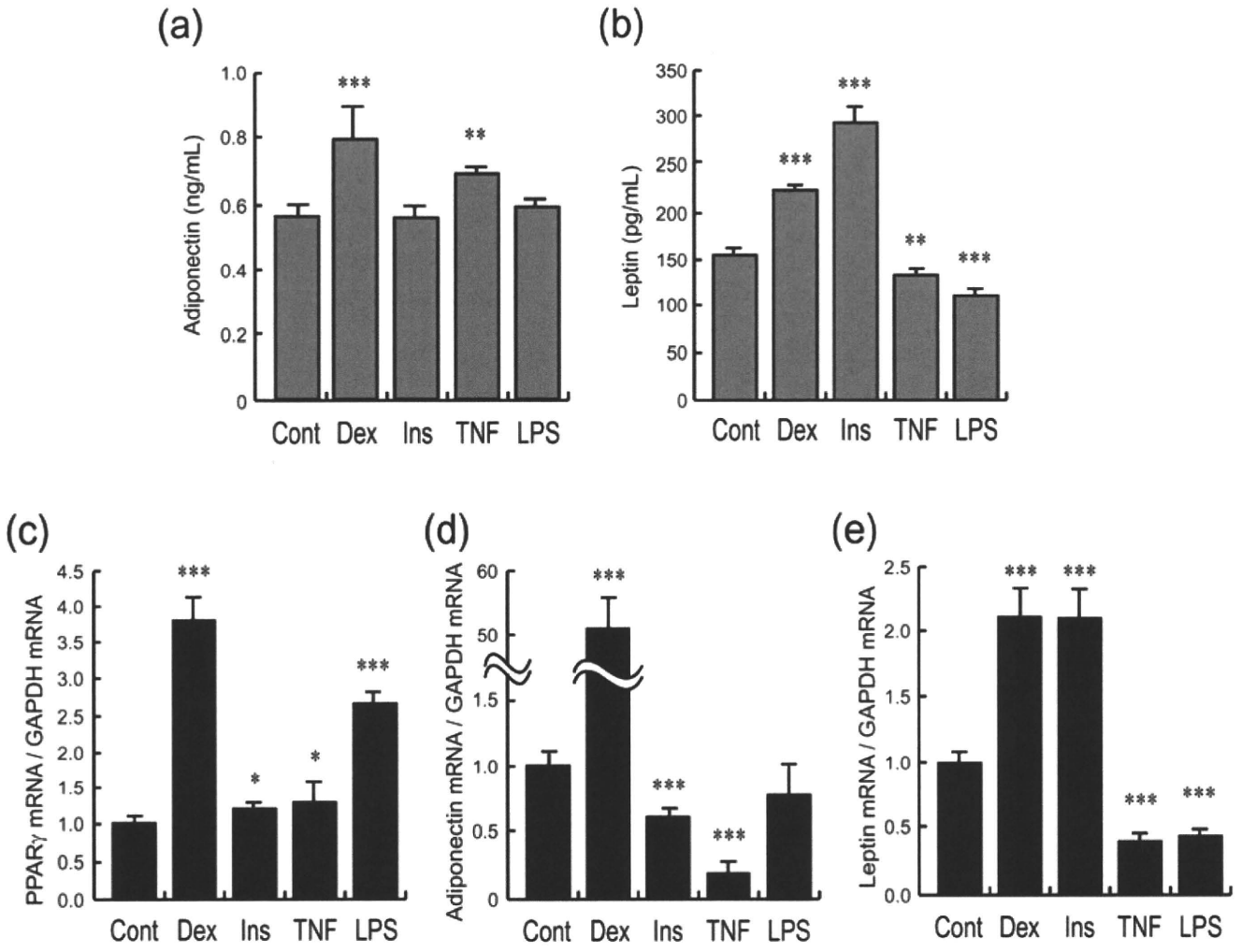

Figure 5 Production of (**a**) adiponectin protein and (**b**) leptin protein, and the mRNA expression of (**c**) peroxisome proliferator-activated receptor (PPAR)γ, (**d**) adiponectin and (**e**) leptin in bone marrow adipose tissue (BMAT) after 1 week in culture, with or without stimulation with several factors for 48 h. The results were analyzed on ELISA and real-time reverse transcription–polymerase chain reaction. (**a**) Adiponectin production in BMAT under all conditions is very low (<0.8 ng/mL). Dexamethasone and tumor necrosis factor-α (TNF-α) increase adiponectin production, while insulin and lipopolysaccharide (LPS) do not. (**b**) Leptin production is detected in cultures without factor stimulation (control). Its production is enhanced with dexamethasone and insulin and inhibited with TNF-α and LPS. (**c–e**) In control cells, mRNA expression of PPARγ, adiponectin and leptin is detected. Dexamethasone significantly enhanced the expression of all the genes. Insulin also promoted expression of PPARγ and leptin, but not expression of adiponectin. TNF-α slightly promoted expression of PPARγ, but clearly inhibited expression of leptin and adiponectin. LPS enhanced expression of PPARγ, but inhibited expression of leptin; it did not affect adiponectin expression. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs control. Cont, control; Dex, dexamethasone; Ins, insulin.

leukocytes did (Fig. 4a,c). Around the fragments, non-lipid containing BMSC also showed BrdU uptake (Fig. 4b,d), but the preadipocytes did not (Fig. 4d).

Adipokine production

Treatment with dexamethasone and TNF-α increased adiponectin production, while insulin and LPS treatment did not. Adiponectin production in BMAT under all culture conditions, however, was <0.8 ng/mL (Fig. 5a). We also found that dexamethasone and insulin increased leptin production, while TNF-α and LPS inhibited it (Fig. 5b).

Adipose tissue-specific gene expression

The addition of dexamethasone to cultures enhanced the expression of PPARγ, adiponectin and leptin. Insulin also increased the expression of PPARγ and leptin, while it inhibited the expression of adiponectin. TNF-α addition promoted the expression of PPARγ, while it inhibited adiponectin and leptin expression. LPS treatment enhanced PPARγ

expression and inhibited leptin expression but had no effect on adiponectin expression (Fig. 5c–e).

BMAT–osteoblast interaction

BMSC were found to actively develop in cultures containing only BMAT fragments (Fig. 6a,c). In contrast, the development of BMSC from the BMAT fragments was inhibited at 1 and 3 weeks when cultured in the presence of osteoblasts (Fig. 6b,c). Adiponectin production in BMAT cultured with or without osteoblasts was not significantly affected and remained very low, while leptin production was inhibited in the BMAT and osteoblast co-cultures (Fig. 6d). BMAT also inhibited BrdU uptake by osteoblasts (Fig. 7a–c) and their expression of the differentiation markers ALP, type I collagen and osteocalcin (Fig. 7d). Interestingly, osteoblasts were found to inhibit BrdU uptake in the mononuclear cells within the BMAT fragments (Fig. 8). Given that these mononuclear cells expressed CD45 (data not shown), these cells were most likely leukocytes.

DISCUSSION

The culture of mature adipocytes has proven to be technically difficult in the past due to their buoyancy in culture medium. To resolve this problem, we have established two systems for culturing isolated mature adipocytes: the ceiling culture[6] and the 3-D collagen gel culture.[18] These methods are useful for studying isolated mature adipocyte behavior, but they do not allow for studying BMAT that contains multiple cell types. In the current study we established a novel culture system for human BMAT fragments embedded in collagen gel. This method maintains mature adipocyte and leukocyte survival for >3 weeks. Furthermore, BMSC, which contain a few preadipocytes and CD44+/CD105+ MSC-like cells, develop around the BMAT fragments. Given that appropriate long-term BMAT culture systems have not been established previously, our method may provide a potentially important approach for studying BMAT biology.

In the present study we found that treatment with dexamethasone drastically increased the number of preadipocytes that developed from BMAT. In contrast, treatment with insulin was not found to affect preadipocyte production. Dexamethasone treatment also enhanced the expression of the adipogenic transcription factor PPARγ to a greater degree than insulin, supporting the findings that BMAT has a lower sensitivity for insulin than subcutaneous adipose tissue.[19,20] Using this same culture system with rat subcutaneous adipose tissue, we have previously shown that insulin significantly increases the number of preadipocytes in the culture.[2] These results suggest that dexamethasone, but not insulin, is a powerful adipogenic modifier of BMAT.

Interestingly, adiponectin production by BMAT cultured under all conditions was very low (<0.8 ng/mL), even though dexamethasone and TNF-α treatment enhanced production. In contrast, insulin and LPS had no effect on adiponectin production. Several studies have demonstrated that dexamethasone and insulin would promote adiponectin production in the subcutaneous and visceral adipose tissues, while TNF-α and LPS would inhibit it.[2,21,22] In our preliminary study we detected adiponectin production at a higher level (11.5 ± 0.2 ng/mL) in cultures of subcutaneous adipose tissues obtained from the same patients whose BMAT materials were used here. These results suggest that BMAT exhibits a different phenotype from that of the visceral and subcutaneous adipose tissues in terms of both adiponectin production and response to dexamethasone and insulin treatment.

Dexamethasone and insulin also promoted leptin production, while TNF-α and LPS inhibited it. The mRNA expression of leptin corresponded well with that of leptin protein under all culture conditions. These responses of BMAT were similar to those of the visceral and subcutaneous adipose tissues.[2,20,21] Leptin has recently been shown to promote osteoblast formation and hematopoiesis, but inhibit adipogenesis.[23,24] These findings suggest that leptin contributes to bone marrow osteogenesis and hematopoiesis.

Dexamethasone also induced high levels of PPARγ expression in BMAT, while insulin only slightly enhanced expression levels. These results are consistent with those reported previously.[19,20] Although TNF-α and LPS reportedly inhibit PPARγ expression in the visceral and subcutaneous adipose tissues,[2] we found that they enhanced it in BMAT, albeit only moderately. Leukocyte-linked cells have also been reported to express PPARγ.[25] In addition, we demonstrated that leukocytes are maintained in our BMAT cultures. Thus, it appears likely that leukocytes within BMAT may be responsible for PPARγ expression induced by TNF-α and LPS.

In the present study we detected the proliferation of BMSC and leukocytes, but not mature adipocytes and preadipocytes. Our previous study showed that mature adipocytes and preadipocytes have the ability to proliferate in the culture system of rat subcutaneous adipose tissue.[2] Although the reasons for this discrepancy are unclear, the following possibilities have been raised: (i) differences between BMAT and subcutaneous adipose tissue; (ii) effects of leukocytes within BMAT; and (iii) species differences between human and rat. To address these important issues, further studies are required.

Hematopoietic cells were reported to require the niche produced by stromal cells for their long-term culture.[26] Dexter culture method is suitable for analyzing the interaction between hematopoietic cells and stromal cells.[27] Our method would provide the microenvironment to analyze the